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EVALUATION OF SOME SOIL FUNGICIDES BY LABORATORY TESTS WITH PHYMATOTRICHUM OMNIVORUM[1]

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INTRODUCTION

Eradication of root rot, caused by *Phymatotrichum omnivorum* (Shear) Duggar, by chemical treatment of the soil is attended by special difficulties. So far as is now known, *P. omnivorum* passes no essential portion of its life history above ground. It spreads from plant to plant by growing from root to root. Infected roots may be located from a few inches to 8 or more feet underground (*30*).[3] Therefore, to have any marked value in eradicating the fungus, a fungicide should be able to operate at much greater depths than materials used to treat diseases of seedlings or other diseases that are controlled at least in part by fungicidal treatment of the surface soil. Experience has in fact shown that materials commonly used as soil fungicides are not effective in eradicating *Phymatotrichum* root rot even from small experimental areas. This has necessitated consideration of other materials, including volatile materials capable of penetrating the soil. The present paper summarizes the laboratory phases of this work, which included development of methods for the evaluation of fungicides as to ability to permeate soil, as well as fungistatic and fungicidal effectiveness. Field trials of some of these materials are reported in an accompanying paper (*3*).

HISTORICAL REVIEW

Many workers with *Phymatotrichum* root rot have reported tests of fungicides. Pammel (*26*) reported field-plot tests of various chemicals. Heavy losses from root rot occurred despite the treatments. Curtis (*1*) attempted to control root rot on alfalfa by application of salt and kerosene.

King (*12*, *13*) reported that further spread of root rot, in small areas in which the disease had just started, had been prevented by soaking them to a depth of 4 feet with a solution of 1 part of 40-percent formalin to 100 parts of water. Taubenhaus and Killough (*31*) found that 1 pint of formalin to 20 gallons of water, applied 2 gallons per square foot, did not control root rot. In further studies by King and Loomis (*15*), the edges of an active root rot area were treated with materials applied in irrigation water. Applications of 3

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[2] The writer is indebted to the late Dr. J. J. Taubenhaus, chief of the Division of Plant Pathology and Physiology, for advice in certain phases of this work; and to the Bayer-Semesan Co., Inc., and the Roessler & Hasslacher Chemical Co. (now R. & H. Chemicals Department, E. I. du Pont de Nemours & Co.), for supplying many of the chemicals tested.

[3] Reference is made by number (italic) to Literature Cited, p. 576.

acre-inches containing 1 percent of carbon disulphide did not greatly reduce root rot; but areas treated with a 2-percent solution of cresylic acid or a 1-percent solution of formaldehyde showed only a few dead plants late in the season. King, Loomis, and Hope (*16*) found that sclerotia of the fungus were inactivated by 30 minutes' exposure to solutions of more than 0.5 percent commercial formalin. Soil saturated with formaldehyde solutions inactivated sclerotia inserted therein; and the fungus was inactivated in sections of infected cotton roots exposed to the same soil within 4 days after the soil had been treated. Treatment of primary centers of infection with formalin was suggested by McNamara (*17*) as effective when tried early in the season. However, Dana (*4*, *32*) reported unsuccessful results from formaldehyde treatments in field plots.

A large-scale test of formaldehyde in a 5-acre infested area near Indio, Calif., was made by the United States Department of Agriculture and the California Department of Agriculture. During May, June, and July, 1930, a 1.25-percent formalin solution was injected by pressure, at the rate of 1 gallon per cubic foot of soil, to a depth of 6 feet. The entire area was treated, with injections made 1 foot apart and 6 gallons of solution introduced at each point. Infected roots and root fragments removed from this treated area 10 months later were found by King and Hope (*14*) to be apparently dead. In 1932 (*20*), however, root rot recurred in certain places in this area. The infested areas were treated again; but the disease again recurred in 1933, in small areas toward the center of the treated region. In 1935 the disease again appeared in a very small spot, and this spot was again treated. Treatment of a second area, near Indian Wells, Calif., at the rate of 1 gallon per cubic foot of soil to a depth of 4 feet, was apparently more successful (*19*).[4]

Following the discovery of the sclerotial stage of the root rot fungus, Neal (*5*), King, Loomis, and Hope (*16*), and others have studied the toxic effect of various solutions on these bodies. King and Hope (*14*) found that fungicides effective against sclerotia or other separate portions of the fungus were not necessarily able to penetrate infected roots and kill the fungus inside such roots. Sclerotia installed inside small blocks made from the roots of plants and buried in soil were not killed by later treatment of the soil with formaldehyde.

Neal (*21*) and Neal, Wester, and Gunn (*24*, *25*) have reported toxicity of ammonia, in solution or gaseous form, against mycelia as well as sclerotia of the root rot fungus, and have suggested the possible utilization of ammonia or ammonium compounds for its control. They stated that 6-percent solutions of ammonium hydroxide applied around large cotton plants in the field apparently killed the fungus in most cases, for when treated roots were removed and observed for 5 days in moist chambers, *Phymatotrichum* growth developed from only one of the eight roots. In 1934, Streets (*27*) stated that heavy applications of ammonium sulphate or ammonium hydrate, diluted to a safe concentration with water, around infected deciduous fruit and nut trees, yielded some promising results. More recently, Neal and Gilbert (*23*) have recommended dilute solutions (2 and 4 percent, respectively) of formaldehyde or ammonia water for eradication of root rot in small centers of infection or in areas

[4] Information partly from letters of Dr. D. G. Milbrath, dated April 25, 1934, and November 2, 1936.

to be planted to ornamentals or fruit trees. Neal and Collins (*22*) have found an initial concentration of about 1,000 p. p. m. of "N as NH_3" necessary to prevent growth from sclerotia in jars of soil.

Several notes on the development of the methods described in the present paper and on the preliminary results obtained have been presented elsewhere (*4*, *5*, *6*, *7*, *8*).

METHODS

The tests were chiefly of two types: (1) Toxicity tests that allowed the materials to act on the fungus without the interposition of soil, and (2) tests of the materials in moist soil to determine their ability to penetrate soil. These latter tests were run by various modifications of the soil-chamber or soil-culture technique. Jars or other containers were filled with unsterilized moist soil, inoculum of the fungus was added, and growth observed through the glass walls of the container. This direct observation is possible because the characteristic strands of *Phymatotrichum omnivorum* grow to a greater extent along the glass-soil surface than into the soil. Only rarely was there any doubt as to the identity of the fungus growth, and in such cases the containers were opened at the end of the experiment and identification checked by microscopic examination of the fungus.

In preliminary experiments set up in December 1928, mixtures of Uspulun, Semesan, and DuPont K–1–P with soil were placed in 1-liter Erlenmeyer flasks, and naturally infected cotton roots were used as inoculum. A more convenient container for the tests was suggested later by the work of Dana (*2*) and the soil-chamber tests described in this paper were run in mason jars instead of in flasks.

In most of the experiments, sclerotial masses were used as inoculum. These masses were taken in all cases from cultures of *Phymatotrichum omnivorum*, the writer's strain No. 24. Large masses produced on various synthetic media (*10*) were removed from the flasks, assembled on moist filter paper in moist chambers, and cut into cubes of approximately 2 or 3 mm. The inoculum used for each point in each jar of a soil-chamber experiment consisted of several portions from white, freshly produced sclerotial masses plus several portions from the older, buff or brown masses. The composite inoculum for every point of inoculation in each jar of a given experiment was thus made directly comparable to the inoculum used in every other jar of the experiment.

Growth starting from the sclerotial inoculum was observed through the glass, the precise extent being determined with a hand lens. Growth continued usually for about 5 to 7 days. Periodic examinations were made, usually after 7 and 14 days; and the longest growth (which was more uniform than the average radius of the colony) from each point of inoculation was noted, rather than the amount of growth within a set period. These maximum figures were averaged to obtain the "mean extent of growth" for each group of cultures as given in the tables.

Particular procedures followed are described below under the various experiments. For convenience, the results of each experiment are arranged with the more effective materials at the tops of the tables; this does not indicate the actual order of trial in the experiments.

SOIL-CHAMBER TESTS OF FUNGICIDES MIXED MECHANICALLY INTO THE SOIL

COMPARATIVE EFFECTIVENESS OF VARIOUS FUNGICIDES

Soil-chamber method A has served to determine for materials that are not too volatile the concentration required in moist soil to inhibit *Phymatotrichum* growth. Weighed or measured amounts of the materials to be tested (calculated as parts per million of the air-dry soil weight) were added to weighed quantities of air-dry Houston black clay surface soil, previously sifted through a ¼-inch mesh wire sieve. (This soil type, which is highly favorable for growth of the root rot fungus and occurs in regions in which the disease is very destructive, was used also in further experiments.) The fungicide was mixed thoroughly into the dry soil by repeated mixing. Sufficient distilled water was added to furnish a final soil moisture of 25 percent on an air-dry soil-weight basis, and incorporated into the soil by further mixing. The moist soil mixture was then distributed in 900-g portions into 1-quart mason jars, usually three for each concentration of each fungicide tested. The soil in the jars was tamped gently into place. Sclerotial inoculum was added the same day or within 2 or 3 days. Each jar was inoculated at two points, on opposite sides, with composite sclerotial inoculum. At each point the inoculum was inserted next to the glass wall of the jar, 2 to 4 cm below the upper surface of the soil mixture, and the soil firmed about it. The jars were covered with the regular mason jar lids, usually without the rubber rings.

The results of two typical experiments by this method are given in table 1. Copper carbonate was the least effective of the materials tested. Both K–1–X and No. 664 inhibited growth at 500 p. p. m. Mercuric chloride was highly fungistatic and prevented all growth even at the lowest concentration, 50 p. p. m.

TABLE 1.—*Comparison of various fungicides when mixed into soil, by method A, using 3 jars for each concentration of each material, and 6 check jars, each jar having been inoculated at 2 places*

Experiment and material tested	Mean extent of *Phymatotrichum* strand growth [1] from the inoculum, in jars with fungicides at concentration of—					
	50 p. p. m.	100 p. p. m.	250 p. p. m.	500 p. p. m.	1,000 p. p. m.	Check
	Millimeters	*Millimeters*	*Millimeters*	*Millimeters*	*Millimeters*	*Millimeters*
Experiment C–4:						
Semesan (30 percent chlorophenol mercury)	41	20	Tr.	0	0	
K–1–X (2 percent ethyl mercury chloride)	31	26	17	Tr.	0	
No. 664 (14 percent nitrophenol mercury)	65	71	17	Tr.	0	
PMA (2.5 percent phenyl mercury acetate)	29	39	21	16	Tr.	
Copper carbonate	102	96	52	92	64	
None (check)						67
Experiment C–5:						
Mercuric chloride	0	0	0	0	0	
Bayer Special 100 × 3 (10 percent chlorophenol mercury)	36	8	Tr.	0	0	
Bayer Special II–6 × 5 (6 percent nitrophenol mercury)	27	15	Tr.	0	0	
K–1–X (2 percent ethyl mercury chloride)	14	18	13	Tr.	0	
Sterocide (4 percent mercury furfuramide)	10	19	16	Tr.	0	
Smuttox (4 percent oxymethylene)	25	20	40	25	33	
None (check)						35

[1] Growth indicated as Tr. consisted of very short, fuzzy growth on the inoculum, less than 10 mm long, and not penetrating soil particles near the inoculum.

INITIAL AND RESIDUAL EFFECTIVENESS OF ORGANIC MERCURY COMPOUNDS

The early laboratory results with the organic mercury compounds were used in planning field-plot tests of some of these materials for the control of root rot on cotton plants. The laboratory results indicated that K–1–X, for example, might be slightly more effective than No. 664 (table 1). In the field trials, however, applications of K–1–X failed to have any effect, while similar weights of No. 664 reduced the spread of root rot (*28*, *29*). The following experiment was planned to determine whether this apparent discrepancy between laboratory and field results might be explained by differences between the initial and the later effectiveness of the materials after contact with moist soil.

The experiment was set up by method A and growth from the inoculum was recorded as usual. After 3 weeks, however, the sclerotial inoculum was removed from the jars. The soil was made up to original weight with distilled water, mixed thoroughly, and placed in new jars. A second set of sclerotial inoculum, which was inserted in these new jars, was exposed to the residual inhibiting effect of the various fungicides which had now been in contact with the soil for 5 weeks. The results of both tests are summarized in table 2.

TABLE 2.—*Initial and residual efficiency of various fungicides when mixed into soil by method A, experiment C7, using 3 jars for each concentration of each material and 6 check jars, each jar having been inoculated at 2 places*

Material	Concentration in the soil	Mean extent of *Phymatotrichum* strand growth in— [1]	
		Initial test	Test after 5 weeks
	Parts per million	*Millimeters*	*Millimeters*
Semesan (DuBay) (30 percent chlorophenol mercury)	50	46	56
	100	39	49
	250	Tr.	0
	500	0	0
	1,000	0	0
DuBay 664 (14 percent nitrophenol mercury)	50	56	52
	100	48	63
	250	49	24
	500	0	0
	1,000	0	0
DuBay 965-D (2 percent ethyl mercury arsenate)	50	77	62
	100	67	36
	250	Tr.	48
	500	0	55
	1,000	Tr.	0
DuBay 971-A (2 percent ethyl mercury phosphate)	50	76	67
	100	63	53
	250	Tr.	41
	500	0	63
	1,000	0	0
K–1–X (2 percent ethyl mercury chloride)	50	77	74
	100	65	50
	250	34	91
	500	0	88
	1,000	0	90
PMA (2.5 percent phenyl mercury acetate)	50	90	79
	100	69	71
	250	43	60
	500	41	46
	1,000	23	23
Formaldehyde, U. S. P.	50	108	52
	100	70	46
	250	90	43
	500	49	14
	1,000	77	52
	5,000	0	59
	10,000	0	0
Gasoline (commercial)	1,000	97	68
	5,000	0	66
	10,000	0	0
None (checks)		104	68

[1] See footnote 1, table 1.

In the first test, the effectiveness of the various materials in inhibiting growth of *Phymatotrichum* was much as in previous experiments. In the second test growth was inhibited at the same concentrations as originally in the Semesan and No. 664 mixtures only. The ethyl mercury compounds were much less effective than in the first tests. K–1–X, which in the first test had prevented *Phymatotrichum* growth completely at 500 p. p. m., in the final test did not impede growth at 1,000 p. p. m. Both 965–D and 971–A were effective at 250 p. p. m. in the initial test, but ineffective at 500 p. p. m. in the final test. Formaldehyde and gasoline also were markedly less effective in the final test.

In the initial test, as in previous tests of fungistatic effectiveness of materials shortly after mixture with soil, the ethyl mercury materials in general were much more effective than would be expected from their actual mercury content as compared to that of chlorophenol and nitrophenol mercury materials. But after exposure to the moist soil this high initial efficiency of the ethyl mercury materials had decreased; and the final, residual effectiveness correlated with the comparative concentration of mercury in the various materials. These results explained the amounts of the materials found to be effective in the first field-plot trials, and suggested more satisfactory rates of application for later trials with ethyl mercury compounds.

In several years of further field-plot trials with organic mercury compounds, repeated large additions of some of these materials greatly reduced the prevalence of root rot, but none was able to eradicate it (*28*, *29*). Part of the difficulty with the mercurials was considered to be their failure to penetrate rapidly and deeply into the soil. In view also of the results reported at this time by Weiss and Evinger (*33*) in the use of naphthalene and alpha-naphthol against *Sclerotium rolfsii*, it seemed advisable to extend tests of possible soil fungicides for use against root rot to include some more highly volatile materials that might penetrate the soil more effectively. Many of these materials were not readily soluble in soil moisture and hence would not be fixed in solution in the particular soil layers in which they might be applied.

SOIL-CHAMBER TESTS OF THE EFFECTIVENESS OF VOLATILE MATERIALS IN PENETRATING SOIL AND PREVENTING GROWTH OF PHYMATOTRICHUM OMNIVORUM

A number of chlorinated hydrocarbons and other volatile materials were tested first by the following method, which indicated whether the materials could penetrate 2 to 4 cm through a standardized moist soil and then inhibit growth of the fungus from standardized sclerotial inoculum.

Soil-chamber method B differed from method A in that the materials to be tested were not mixed mechanically with the soil but were placed on the surface of the soil after the inoculum had been inserted in the jars. The quantity of material added was calculated as parts per million of the air-dry weight of soil in the jar. Liquid materials were poured rapidly directly on the soil surface; solid materials were placed in piles on the surface. The jar was then quickly sealed, using the rubber ring. Duplicate jars were used for each concentration of each material tested. Each jar was inoculated at two points, four sets of

inoculum thus being exposed to each concentration. Four jars were used for the checks in each series.

TABLE 3.—*Fungistatic effectiveness of various materials when placed on the surface of soil in soil chambers, in preventing growth from sclerotial inocula located 2 to 4 cm below, method B*

Experiment and material tested	Mean extent of *Phymatotrichum* strand growth from the inocula in jars where materials were applied at the rate of—				
	100 p. p. m.	500 p. p. m.	1,000 p. p. m.	2,000 p. p. m.	Check
	Millimeters	*Millimeters*	*Millimeters*	*Millimeters*	*Millimeters*
Experiment C–8–a:					
Pentachlorethane	0	0	0	0	
Dichlorethylene	16	0	0	0	
Trichlorethylene	18	0	0	0	
Perchlorethylene	38	0	0	0	
Dichlormethane	70	40	0	0	
Acetone	58	59	49	91	
None (checks)					78
Experiment C–8–b:					
Xylene	0	0	0	0	
Carbon disulphide	0	0	0	0	
Benzene	19	0	0	0	
Kerosene saturated with paradichlorobenzene	30	0	0	0	
Ethylene dichloride	40	9	0	0	
Cresylic acid	56	25	17	19	
Sulphur chloride	81	75	85	71	
None (checks)					65
Experiment C–9:					
Turpentine	0	0	0	0	
Paradichlorobenzene	0	0	0	0	
Naphthalene	0	0	0	0	
Kerosene (commercial)	96	0	0	0	
Carbon tetrachloride	94	8	0	0	
Iodine	40	31	0	0	
Hexamethylenetetramine	41	69	58	31	
Thymol	55	31	41	43	
Alpha-naphthol	74	79	40	53	
Petroleum (lubricating) oil	58	55	74	63	
None (checks)					53

The results of three experiments by this method are listed in table 3. Pentachlorethane, xylene, carbon disulphide, turpentine, paradichlorobenzene, and napthalene completely inhibited growth from the sclerotial inoculum, even at 100 p. p. m. Five other materials allowed growth at this concentration but prevented it at higher concentrations. These materials seemed promising and were studied further, as described below.

EFFECTIVENESS OF VOLATILE MATERIALS AFTER PENETRATING DIFFERENT DEPTHS IN THE SOIL

Soil-chamber method C made it possible to determine the extent to which volatile materials would penetrate moist soil for more considerable distances, as shown by the effect on sclerotial inoculum in soil chambers. Method C was the same as method B, except that instead of using two sets of sclerotial inoculum toward the top of the jar, six sets were used, a pair toward the bottom, a pair toward the middle, and a pair toward the top. A diagram of this set-up is given in figure 1, *A*.

In filling a jar, two masses of inoculum were placed on the bottom at opposite sides. Half of the soil (by weight) for the jar was added and compacted to the usual density. Two more masses of inoculum were then placed on this soil and next to opposite walls of the jar. Most

of the rest of the soil was then added and compacted, the final two masses of inoculum inserted, and finally the remaining soil added and compacted. After this set-up was complete, the material to be tested was placed on the surface, and the jar lid sealed, using the rubber ring.

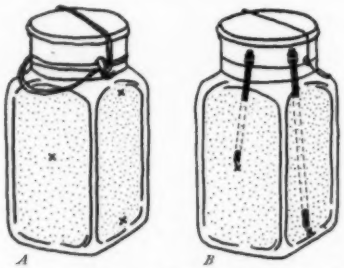

FIGURE 1.—Soil-chamber methods for testing penetration of fungicides through soil. *A*, Diagram of set-up by method C. Fungus inoculum was inserted in soil at points indicated by X marks as the jars were filled, and fungicides were then applied on surface of soil. *B*, Diagram of set-up by method D. Four glass tubes, capped with rubber tubing and glass rod, were installed in the soil. The fungicides were added, and after various periods the inoculum was pushed down the tubes to the points indicated, to determine residual fungistatic effectiveness.

TABLE 4.—*Fungistatic effectiveness of various materials, when placed on the surface of soil in soil chambers, in preventing growth from sclerotial inoculum located at different depths, method C*

Experiment and material tested	Rates of application [2]	Mean extent of *Phymatotrichum* strand growth,[1] from—		
		Upper inoculum	Middle inoculum	Bottom inoculum
	Parts per million	*Millimeters*	*Millimeters*	*Millimeters*
Experiment C-10:				
Pentachlorethane	100 to 2,000	0	0	0
Carbon disulphide	100 to 2,000	0	0	0
Turpentine	100 to 2,000	0	0	0
Paradichlorobenzene	100 to 2,000	0	0	0
Dichlormethane	100	33	38	40
	500	0	7	0
	1,000 and 2,000	0	0	0
Naphthalene flakes	100	0	11	12
	500	0	10	36
	1,000	0	5	24
	2,000	0	0	4
None (checks)		22	27	29
Experiment C-11:				
Tetrachlorethane	100 to 2,000	0	0	0
Xylene	100 to 2,000	0	0	0
Perchlorethylene	100 to 2,000	0	0	0
Trichlorethylene	100 to 2,000	0	0	0
Dichlorethylene	100 to 2,000	0	0	0
Benzene	100	18	30	26
	500 to 2,000	0	0	0
Kerosene	100	10	23	35
	500	0	0	8
	1,000	0	0	5
	2,000	0	0	0
None (checks)		33	40	39
Experiment C-14:				
Chloroform	100	55	50	56
	500 to 2,000	0	0	0
Ether	100	61	41	65
	500	66	54	58
	1,000	41	51	41
	2,000	Tr.	Tr.	Tr.
None (checks)		49	61	58

[1] See footnote 1, table 1.
[2] Materials in these experiments were all tested at 100, 500, 1,000, and 2,000 parts per million.

The upper inocula were at an average depth of about 15 mm below the surface of the soil, the middle inocula at about 75 mm, and the bottom inocula at about 134 mm. The various fungicides could prevent growth from the inoculum only after penetrating the soil to the depths mentioned. While such penetration through the moist soil materials in the jar would not prove that the fungicide would penetrate soil in the field, lack of penetration would strongly indicate the improbability of such penetration through soil under natural conditions.

TABLE 5.—*Fungistatic effectiveness of organic mercury compounds, formaldehyde and ammonia, as compared to other materials when placed on the surface of soil in soil chambers, in preventing growth from sclerotial inoculum located at different depths, method C*

Experiment and material tested	Rates of application	Mean extent of *Phymatotrichum* strand growth, from—		
		Upper inoculum	Middle inoculum	Bottom inoculum
	Parts per million	*Millimeters*	*Millimeters*	*Millimeters*
Experiment C–12:				
Pentachlorethane	100	0	0	0
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
Xylene	100	0	0	0
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
Paradichlorobenzene	100	1	0	3
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
DuBay 971–A (2 percent ethyl mercury phosphate).	100	28	28	40
	500	23	23	24
	1,000	15	10	14
	2,000	0	0	14
Semesan (30 percent chlorophenol mercury)	100	26	26	33
	500	36	38	45
	1,000	26	35	70
	2,000	0	29	54
Formaldehyde (U. S. P. 37 percent)	500	29	28	39
	1,000	26	30	39
	2,000	26	31	24
	4,000	14	26	36
Ammonia (NH_4OH, specific gravity, 0.90)	500	33	34	33
	2,000	1	49	43
	5,000	0	31	48
	10,000	0	3	40
None (checks)		50	44	53
Experiment C–15:				
Pentachlorethane	100	0	0	0
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
Ethyl chloride	100	53	56	53
	500	38	36	36
	1,000	0	0	0
	2,000	0	0	0
New Improved Ceresan (5 percent ethyl mercury phosphate).	100	45	63	60
	500	8	34	48
	1,000	0	29	61
	2,000	0	0	20
DuBay 1153–A (2.5 percent ethyl mercury phosphate).	100	40	50	50
	500	8	21	59
	1,000	0	31	51
	2,000	5	20	29
None (checks)		48	49	55

TABLE 6.—*Fungistatic effectiveness of smaller quantities of certain materials, when placed on the surface of soil in soil chambers, in preventing growth from sclerotial inoculum located at different depths, method C, experiment C–21*

Material tested	Rates of application	Mean extent of *Phymatotrichum* strand growth, from—		
		Upper inoculum	Middle inoculum	Bottom inoculum
	Parts per million	*Millimeters*	*Millimeters*	*Millimeters*
Industrial xylol	50	[1] 15	[1] 8	[1] 8
	75	0	0	0
	100	0	0	0
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
Tetrachlorethane	50	0	0	0
	75	[1] 16	[1] 21	[1] 23
	100	0	0	0
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
Industrial gasoline	50	0	0	0
	75	50	49	43
	100	40	30	31
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
None (checks)		41	41	39

[1] *P. omnivorum* growth in 1 of the 2 jars only.

Three experiments by this method are summarized in table 4. Nine of the materials were highly efficient, even at the lowest rate of application, inhibiting growth from even the bottom inoculum. Naphthalene prevented growth from the upper inoculum at 100 p. p. m., but was not completely effective against the deep inoculum even at 2,000 p. p. m. Ether penetrated the soil evenly but was able to inhibit *Phymatotrichum* development only when present at more than 1,000 p. p. m., while chloroform was effective at concentrations above 100 p. p. m.

In two experiments, C–12 and C–15 (table 5), organic mercury compounds, formaldehyde, and ammonia were compared with other materials. In experiment C–12, the Semesan and DuBay 971–A dusts were placed in appropriate amounts on the surface of the soil. More than half of the usual allotment of water for the soil of each jar had been reserved from the original mixing and was now added slowly over a period of 3 hours. The water trickled down fairly rapidly through the loose soil in the jars, and carried with it portions of the dusts. In experiment C–15, the New Improved Ceresan and DuBay 1153–A were merely placed on the moist surface soil, in the same way as the other materials, and the tops of the jars were sealed immediately. The ethyl chloride was handled in a flask cooled in a freezing mixture and dispensed rapidly with a pipette cooled in liquid ethyl chloride in the flask. The jars were sealed immediately after the ethyl chloride was placed on the surface of the soil; nevertheless, some must have escaped.

There were marked differences in the effectiveness of the various materials (table 5). Pentachlorethane and xylene, even at the lowest rates of application, completely inhibited growth of *Phymatotrichum* from the bottom inoculum. Paradichlorobenzene was not completely

effective at the lowest rate. Ethyl chloride penetrated the soil thoroughly but was effective only at concentrations above 500 p. p. m. All the other materials in experiments C–12 and C–15 were ineffective at the lower depths in the soil, although when present in relatively large amounts they inhibited growth from the upper inoculum. None of the four organic mercuries, irrespective of the method of application, inhibited growth from the bottom inoculum. The ethyl mercury-phosphate mixtures were more effective than formaldehyde in preventing growth from the upper inoculum.

Another experiment on the fungistatic effectiveness of certain materials was run by method C as in the experiments above, except that lower concentrations of the materials were tested. The results (table 6, experiment C–21), indicate that industrial gasoline is less effective against *Phymatotrichum* than is industrial xylol or tetrachlorethane. Xylol and tetrachlorethane were of approximately equal effectiveness. With each, 100 p. p. m. was uniformly effective in inhibiting growth of the fungus, while in a single jar receiving lower rates, there was definite although short growth from the inoculum. These results suggest that the rate of 100 p. p. m. of air-dry soil weight, used in most of the earlier tests, is probably near the minimum concentration required, even with the more effective of the fungicides tested, to inhibit growth of *P. omnivorum*.

It may be noted that the results obtained in these tests are not directly comparable to those obtained by other workers with other types of soil or with fungicides applied in dilute aqueous solution to drench the entire soil mass. In extensive studies on penetration of soil by fungicides, Hunt, O'Donnell, and Marshall (*11*) found that formaldehyde in dilute solutions percolated as rapidly as water through tubes of Manor and Leonardtown loams. Milbrath (*18*) similarly found that 1-percent solutions of formaldehyde passed through tubes of soil materials. Yet in the present work (table 5) undiluted United States Pharmacopoeia formaldehyde added on the surface of the heavy Houston soil in the jars, in sufficient quantity to furnish 4,000 p. p. m. of the air-dry weight of the soil, lowered but did not prohibit *Phymatotrichum* growth from inoculum located only 15 mm below the surface. The weight of formaldehyde added to furnish 4,000 p. p. m. was more than would be needed to furnish a 1-percent solution, as used by Milbrath, to saturate all the soil in the jar.

INITIAL AND RESIDUAL FUNGISTATIC EFFECTIVENESS OF PENTACHLORETHANE AND DUBAY 1153–A AFTER PENETRATING DIFFERENT DEPTHS IN SOIL

In the tests summarized above, pentachlorethane and several other chemicals were much more effective than the organic mercury materials. It was important to find whether this would be true also after the materials had been in contact with the moist soil for some time, and whether an organic mercury material might penetrate the soil by diffusing gradually downward.

An experiment was set up in which pentachlorethane and DuBay 1153–A were tested at rates ranging from 100 to 2,000 p. p. m. Pentachlorethane was placed on the surface of the soil, while the organic mercury dust was placed in a thin layer one-fourth to one-half inch below the surface of the moist soil. Two jars were used for

each rate of application of each material, in each of four series. Six jars for each series were used as checks. Series 1 and 2 were set up in soil chambers by method C, to determine the immediate fungistatic effectiveness of the materials.

Soil-chamber method D was used for series 3 and 4, to determine the residual fungistatic effectiveness. With this method the inoculum was not inserted until several weeks after the fungicides had been applied. As the jars were filled with soil, four glass tubes of 4-mm inside diameter were so placed that two reached nearly to the bottom of opposite walls of the jar, and the other two reached to the middle of alternate walls. Each tube was capped with a small piece of rubber tubing, which in turn was plugged with a bit of glass rod. The soil was carefully filled in and packed around these tubes, leaving the capped upper ends exposed (fig. 1, *B*). The fungicidal materials were applied at the beginning of the experiment just as in series 1 and 2. After 4 weeks for series 3, and 8 weeks for series 4, the jars were opened one by one, the glass plug on the upper end of each tube pulled out, and a mass of sclerotial inoculum pushed (by means of a slender glass rod) down through the tube and into contact with the soil. The glass rod was then withdrawn, the glass plug replaced to prevent later diffusion of the fungicide down the tubes, and the top of the jar replaced as soon as all four of the inocula had been inserted. No upper inoculum was used. This method allowed inocula to be placed deep in soil, already exposed to fungicides for considerable periods, without disturbing the soil and possibly allowing escape of volatile materials.

The jars of series 2 were opened and the soil surfaces exposed to gentle fanning for 5 hours on the second day and again on the third day after the beginning of the experiment. The effectiveness of the fungicides was evidently not affected by the short periods during which these jars were open; so series 1 and 2 both show initial effectiveness of the materials, and can be compared to series 3 and 4 representing effectiveness of the materials after 4 and 8 weeks, respectively.

As usual in soil-chamber tests, the pentachlorethane additions were completely effective in the lowest concentration tested against all six masses of inoculum in every jar, in series 1 and 2 (table 7). But after 4 or 8 weeks' contact with the soil the 100 p. p. m. concentration no longer exerted an inhibitive effect and 500 p. p. m. reduced but did not prevent growth. After 4 weeks the fungistatic effectiveness was only a fifth to a tenth of the original high value. Apparently an equilibrium was reached by this time, since the material was equally effective after an additional 4 weeks.

DuBay 1153–A caused only partial reduction in growth from the upper inoculum of those jars of series 1 and 2 that received 2,000 p. p. m., and was equally ineffective after 4 and 8 weeks. The ethyl mercury phosphate apparently did not diffuse downward sufficiently during the entire time to reduce fungus growth. But the aberrant results with this material in series 3 and 4 at 500 p. p. m. cannot be explained, and suggest the need of further test of the possibility of delayed penetration of soil by ethyl mercury phosphate.

TABLE 7.—*Initial and residual effectiveness of pentachlorethane and DuBay 1153–A (2.5 percent ethyl mercury phosphate) in preventing growth from sclerotial inoculum located at different depths, experiment C–16*

Series No. and method	Material tested	Rates of application	Mean extent of *Phymatotrichum* strand growth from—		
			Upper inoculum	Middle inoculum	Bottom inoculum
		Parts per million	*Millimeters*	*Millimeters*	*Millimeters*
1. Inoculum placed in jars as filled, jars not opened later.	Pentachlorethane	100	0	0	0
		500	0	0	0
		1,000	0	0	0
		2,000	0	0	0
	DuBay 1153–A	100	53	53	53
		500	48	46	55
		1,000	40	46	54
		2,000	5	41	51
	None (checks)		48	41	53
2. Inoculum placed in jars as filled, jars opened 5 hours each on third and fourth day.	Pentachlorethane	100	0	0	0
		500	0	0	0
		1,000	0	0	0
		2,000	0	0	0
	DuBay 1153–A	100	50	41	56
		500	44	44	63
		1,000	36	38	51
		2,000	8	31	61
	None (checks)		49	47	54
3. Inoculum inserted through tubes after 4 weeks.	Pentachlorethane	100	----------	55	55
		500	----------	16	20
		1,000	----------	0	0
		2,000	----------	0	0
	DuBay 1153–A	100	----------	70	60
		500	----------	0	0
		1,000	----------	26	28
		2,000	----------	43	59
	None (checks)		----------	58	45
4. Inoculum inserted through tubes after 8 weeks.	Pentachlorethane	100	----------	75	60
		500	----------	9	8
		1,000	----------	0	0
		2,000	----------	0	0
	DuBay 1153–A	100	----------	39	33
		500	----------	0	0
		1,000	----------	80	79
		2,000	----------	81	69
	None (checks)		----------	62	51

EFFECTIVENESS OF VARIOUS MATERIALS WITH INFECTED COTTON ROOTS USED AS INOCULUM

In the previous tests of volatile materials in soil chambers, portions of sclerotial masses of the fungus produced in artificial culture were used as inoculum. An experiment was planned to find whether the materials would be equally effective if infected roots were used instead. For this work cotton roots only partially decayed by root rot were collected on October 3, 1933. Since earlier work had shown that the fungus is most active near the advancing edge of the invaded portion of a root, each root was cut down to a piece 8 to 10 cm long, with most of the disintegrated lower portion removed and only about 3 cm of the upper uninvaded portion left above the infected area. Two roots were placed on the bottom of each mason jar, which was then filled with moist Houston soil. Six jars were used for each concentration of each material tested. Pentachlorethane, tetrachlorethane, and xylene were applied on the surface of the soil, at rates of 100, 1,000, and 2,000 p. p. m. of the air-dry soil weight.

Pentachlorethane prevented any *Phymatotrichum* growth from the infected roots. In both the tetrachlorethane and xylene series there was some strand development in one of the six jars receiving only

100 p. p. m. Profuse growth occurred in 9 of the 14 check jars with untreated soil. While pentachlorethane was apparently as effective in preventing growth from the infected roots as previously in preventing growth from sclerotial inoculum, tetrachlorethane and xylene were slightly less effective with the roots than with the sclerotial inoculum.

COMPACTNESS OF SOIL IN JARS AS AFFECTING PENETRATION AND FUNGISTATIC EFFECTIVENESS OF PENTACHLORETHANE

An experiment was set up by method C as outlined above, except for variations in the compactness of the soil. One series of soil chambers was prepared as usual with 900 g of moist Houston soil per 1-quart jar, another series with 1.2 times this weight, and a third with 1.4 times this weight. For the third series, the soil was pounded into the jars with pestles, compacting it to a bricklike mass. Pentachlorethane was added on the surface of the soil in amounts to furnish 100, 250, 500, and 1,000 p. p. m., respectively, of the air-dry weight of the 900 g of soil used in the usual set-up. The pentachlorethane inhibited growth from sclerotial inoculum at all three depths in every jar, while good growth occurred in the untreated jars of most of the series. In the check jars with the most compact soil, however, strands developed from only two of the four middle inocula, and from only one of the four bottom inocula. Absence of growth in pentachlorethane-treated jars of this set was therefore not so significant as in the other series.

In another experiment with soil compacted to 1.5 times the usual density, strand growth developed from one of the four middle inocula of the jars receiving 300 and 500 p. p. m. of pentachlorethane, and from some inocula at all depths in jars receiving 100 and 200 p. p. m.

In these tests, pentachlorethane readily permeated Houston soil somewhat more compact than that used in most of these laboratory tests, but did not penetrate uniformly through soil compacted to about 1.5 times this density.

APPLICATIONS OF VOLATILE FUNGICIDES IN CLOSED AS COMPARED TO OPEN SOIL CHAMBERS AND ON SURFACE OF SOIL AS COMPARED TO INSERTION IN HOLES

The several lines of experiment discussed above had shown that pentachlorethane, tetrachlorethane, and xylene, applied on the surface of soil in closed jars, even at very low concentrations, penetrated the soil and inhibited growth of *Phymatotrichum*. Obviously, conditions of this sort would not exist in the field, where the fungicides might readily evaporate and be lost in the air. Several experiments were therefore set up to determine the effectiveness of these volatile materials when used in open jars.

In the experiment summarized in table 8, each of the five series of jars included duplicate jars (set up with upper, middle, and bottom inoculum as described for method C above) for each of the four concentrations of pentachlorethane used. After pentachlorethane was added, the jars of series 1 were left open continuously; those of series 2 were closed for 2 hours; those of series 3 for 18 hours; and those of series 4 for 48 hours, and then left open afterwards. The jars of series 5 were closed continuously. Jars of series 1 to 4 were weighed at the beginning of the experiment and at intervals of 2 to 3 days thereafter, and water lost by evaporation was replaced.

TABLE 8.—*Fungistatic effectiveness of pentachlorethane in open as compared with closed jars, in preventing growth from sclerotial inoculum located at different depths, method C, experiment C–17*

Series No. and treatment of the jars	Rates of application	Mean extent of *Phymatotrichum* strand growth from—		
		Upper inoculum	Middle inoculum	Bottom inoculum
	Parts per million	*Millimeters*	*Millimeters*	*Millimeters*
1. Open continuously	100	68	56	56
	500	39	48	50
	1,000	0	0	0
	2,000	0	0	0
2. Closed for 2 hours, open thereafter	100	63	70	54
	500	0	28	66
	1,000	0	0	0
	2,000	0	0	0
3. Closed for 18 hours, open thereafter	100	35	33	56
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
4. Closed for 48 hours, open thereafter	100	0	0	40
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0
5. Closed continuously	100	0	0	0
	500	0	0	0
	1,000	0	0	0
	2,000	0	0	0

Pentachlorethane was not so effective in the opened jars as in the series that remained closed (table 8). In the closed jars, pentachlorethane was completely effective, even at the lowest concentration, in preventing growth from the bottom inoculum. In the jars closed for 48 hours before being opened, growth occurred only from the bottom inoculum with pentachlorethane applied at 100 p. p. m. In the jars closed for only 18 hours, the 100-p. p. m. treatment did not inhibit growth from inoculum at any of the depths; and in the jars left open continuously or closed for only 2 hours before being opened, the 500-p. p. m. treatment was not effective.

Another experiment, summarized in part in table 9, was set up in 2-quart mason jars with 1,800 g of moist soil per jar. Pentachlorethane, tetrachlorethane, and xylene were tested, each in three separate series. In the first series, the fungicides were poured on the surface of the soil in the jars; in the second series the materials were poured into small holes, a half inch in diameter and 3 inches deep, punched in the center of each jar. The holes were filled in tightly with soil as soon as the fungicides had been inserted. These two series, and the untreated check jars, were left open and exposed to a constant breeze from an electric fan. At intervals of 2 to 3 days, each jar was weighed and the water lost by evaporation was replaced. There was apparently a fairly rapid loss of the fungicides from these jars. A strong odor was perceptible over the jars during the early days of the experiment. By the end of the first week, however, the odor was much weaker, and after 2 weeks it was imperceptible.

In a third series, not included in table 9, the jars were sealed as usual when method C was used. All three fungicides were effective in preventing growth from even the bottom inoculum, with the lowest concentration used, while profuse growth occurred in the jars of

untreated soil. Thus this series proved that the materials were able to penetrate the 165 mm of moist soil to the bottoms of these deeper, 2-quart jars.

TABLE 9.—*Relative fungistatic effectiveness of application of volatile materials on the surface of soil as compared to application in holes 3 inches deep in the soil, in open jars exposed to constant fanning, experiment C–20*

Methods of application	Material tested	Rates of application	Mean extent of *Phymatotrichum* growth from—		
			Upper inoculum	Middle inoculum	Bottom inoculum
		Parts per million	*Millimeters*	*Millimeters*	*Millimeters*
On surface of soil in jars	Pentachlorethane	100	24	54	80
		500	55	64	[1] [2] 34
		1,000	[2] 13	39	[1] 30
		2,000	0	[1] [2] 33	[1] 61
	Tetrachlorethane	100	34	58	71
		500	[2] 8	56	53
		1,000	24	86	59
		2,000	[1] [2] 15	58	104
	Xylene	100	41	66	59
		500	48	71	65
		1,000	[2] 18	74	58
		2,000	[1] [2] 11	69	70
In holes 3 inches deep in the soil	Pentachlorethane	100	[1] 38	49	76
		500	[1] 86	0	[1] [2] 28
		1,000	[1] 70	0	0
		2,000	[1] [2] 66	0	0
	Tetrachlorethane	100	39	58	64
		500	84	0	0
		1,000	[1] 134	0	0
		2,000	[1] [2] 23	0	0
	Xylene	100	80	74	84
		500	[1] 71	0	[1] [2] 16
		1,000	[1] 86	0	0
		2,000	[1] 61	0	0
None (checks)			49	57	68

[1] No growth recorded for first 7 days.
[2] Growth from only 1 or 2 of the 4 sclerotial masses.

The open jars exposed to constant fanning (table 9) yielded quite different results. None of the materials permanently inhibited fungus growth. Materials poured on the surface did not inhibit growth from the middle or bottom inoculum. Growth from the upper inoculum was inhibited only temporarily, except with pentachlorethane which held back growth in many jars for at least a week and permanently inhibited growth from the upper inoculum with 2,000 p. p. m. In the series in which the materials were inserted in holes 3 inches deep, on the other hand, the greater effect was observed on the deeper rather than on the upper inoculum. Growth was inhibited for the first week in almost all jars except those with the lowest concentrations of the fungicides. After this temporary effect, profuse growth was secured from most of the upper masses of inoculum. But the middle inoculum, which was nearest to the depth at which the fungicides had been inserted, did not grow; and there was growth from the deep inoculum only in one side of one jar at 500 p. p. m. of pentachlorethane, and from one side of a jar at 500 p. p. m. of xylene. During the second and third weeks, fungus strands from the upper inoculum spread down into the soil and around the middle and lower masses of inoculum, which still failed to grow and apparently were dead.

Since a concentration of only 100 p. p. m. of any of these fungicides was sufficient to inhibit growth of the fungus at any depth in the closed jars of this experiment, it is obvious that this concentration was not maintained in the open jars of either series. The repeated alternate wetting of the surface soil in these jars, followed by rapid drying from the fanning, resulted in frequent deep cracking of the soil and allowed unusual opportunity for rapid evaporation of the fungicides. This explains the results in the series in which the fungicides had been placed 3 inches deep in the soil. Early inhibition of growth from the upper inoculum here was presumably due to the presence, near the surface, of nontoxic concentrations of the materials, which were later lost by evaporation, and growth from the still viable inoculum then occurred. But deeper in the jars, higher concentrations must have been present long enough to kill the sclerotial masses before the fungicides were dissipated into the air.

This experiment made possible rather close comparison between pentachlorethane, tetrachlorethane, and xylene. Particularly in the first series with opened jars, pentachlorethane appeared slightly the most effective, both in inhibition of growth during the first week and in final prevention of growth. Tetrachlorethane and xylene appeared of almost exactly equal value.

These experiments suggest that the probable effectiveness of volatile fungicides in the field will be increased if, instead of the liquids being merely poured on the surface soil, they are inserted in holes deep enough to prevent too rapid loss from the surface, or confined by a cover of some sort for 18 to 48 hours. In preliminary field trials (*9*) volatile fungicides have accordingly been applied in holes.

TESTS OF THE TOXICITY OF VOLATILE MATERIALS

Most of the soil-chamber tests so far mentioned served to indicate only the fungistatic effectiveness of materials, that is, whether materials placed on top of the soil were able to prevent the fungus from growing. Further evidence of the probable value of materials as soil fungicides could be obtained by determining whether the materials were able to kill the fungus. Some toxicity tests were therefore run, separately from the soil-chamber studies and without considering the relative penetrating power of the materials in the soil.

In one experiment of this sort, bits of sclerotial masses from flask cultures of *Phymatotrichum omnivorum* were planted on the surface of agar slants. The tubes were sealed in 2-quart mason jars with the various materials to be tested. Sufficiently volatile materials could pass through the air and the cotton plugs in the tubes, and act on the fungus. The tubes were exposed to three arbitrary concentrations of materials: 0.144, 0.72, and 2.88 g per 2-quart jar. (These weights would provide concentrations respectively of 100, 500, and 2,000 p. p. m. if added to 1,800 g of moist Houston soil, sufficient to fill 2-quart jars. The concentrations were thus planned to be comparable to concentrations used in soil-chamber tests, but obviously only to the extent that moist soil in the jar in the soil-chamber test did not lower the effectiveness of a particular material by preventing it from diffusing, or increase effectiveness of a more pervasive material by filling part of the space with an immiscible volume of soil.) After exposure in the jars for periods respectively of 1, 7, and 14 days,

three tubes were removed for each concentration of each material, aerated for 1 hour in front of an electric fan, and incubated for several months to observe possible *Phymatotrichum* growth.

TABLE 10.—*Preliminary test of toxicity of volatile materials, confined in mason jars, to sclerotia of Phymatotrichum omnivorum exposed on agar slants for the periods indicated, experiment E–1*

Material tested	Growth (+) or lack of growth (−) of the fungus after exposure, for the number of days shown, to the indicated weights of material per 2-quart jars								
	0.144 g			0.72 g			2.88 g		
	1 day	7 days	14 days	1 day	7 days	14 days	1 day	7 days	14 days
Pentachlorethane	−	−	−	−	−	−	−	−	−
Ammonia	−	−	−	−	−	−	−	−	−
Xylene	[1] +	−	−	−	−	−	−	−	−
Carbon disulphide	[1] +	−	−	−	−	−	−	−	−
Formaldehyde	+	−	−	[1] +	−	−	[1] +	−	−
Paradichlorobenzene	+	+	+	+	[1] +	−	+	−	−
Turpentine	+	+	+	+	+	+	+	+	+
None (check)	+	+	+	+	+	+	+	+	+

[1] Growth in only 1 of 3 slants.

Marked differences were found (table 10) between the various materials. Pentachlorethane and ammonia prevented any further growth of the fungus, even at the lowest concentration and for the shortest period of exposure; while paradichlorobenzene was only slightly toxic and turpentine did not prevent growth even during the period of treatment.

In the series just described, weakly fungicidal materials absorbed in the agar substratum might possibly have prevented later growth even from uninjured inocula. The exposure to carbon disulphide, for example, had discolored the agar; and the ammonia had produced a fine precipitate in the agar.

Later toxicity tests were accordingly run by a modified procedure. In this procedure, bits of sclerotial masses of *Phymatotrichum omnivorum*, resting on small wads of moist absorbent cotton in culture tubes plugged with nonabsorbent cotton, were exposed to the fumes of volatile materials confined within 2-quart jars. After specified intervals, the tubes were removed from the jars, aerated in front of an electric fan for 1 hour, and each sclerotial piece was then transferred aseptically to an agar slant. The fungicide itself could be carried over to the agar slant only by passing through the air and the cotton plug and depositing on or in the bit of inoculum. The entire procedure was carried out aseptically, so far as the inoculum was concerned, so that no contaminations entered and observations on large numbers of cultures could be made rapidly and with little trouble. There was no necessity for aseptic precautions with regard to the mason jar containing the fungicide.

In the following experiments the culture tubes used inside the jars each contained a wad of about 0.2 g of absorbent cotton, moistened with 3 cc of distilled water. These tubes were autoclaved, then a piece about 2 by 1 by 1 mm of a fresh, white sclerotial mass of *Phymatotrichum omnivorum* was deposited aseptically in each tube. Three or

more tubes, for each harvest of each concentration of each material to be tested, were placed in 2-quart mason jars. The material to be tested was then introduced into the jar, to the side of the tubes, care being taken to prevent contact with the cotton plugs. Immediately afterward the jar was sealed tightly and tilted just enough to facilitate entrance of heavy gases into the tubes, but not enough to allow the liquid or solid material to come in contact with the plugs. After the respective periods, the jar was opened and the tubes removed quickly. The sclerotial masses were transferred to slants of synthetic medium No. 135 (*10*). The slants were incubated for 3 to 4 months and examined periodically.

To test the possibility that the period of observation in these toxicity tests might not be long enough, certain slants were held longer, in some cases for more than 8 months. In one series, the inoculum was transferred at approximately monthly intervals from the old agar slants to fresh slants, to dispose of traces of fungistatic substances. Another set of sclerotial masses, of which some had been treated with pentachlorethane and others with paradichlorobenzene, were taken from the agar slants on which they had failed to grow, sliced into smaller pieces to expose fresh surfaces, and distributed to new agar slants. None of these special treatments resulted in growth from inocula that had not previously produced growth. Those that failed to grow within the usual period of incubation probably were dead.

In experiments E–2, E–3, and E–4, a single jar was used for the three periods of exposure to a particular concentration of a material. Thus in experiment E–2, nine tubes were placed in the jar with the material; the jar was opened after 24 hours, three tubes withdrawn, and the jar quickly sealed again; it was opened 48 hours later for the second harvest; and after 7 days for the final harvest. Some of the volatile material was lost each time the jar was opened, even though it was closed again within 7 or 8 seconds. This loss lowered progressively the concentration of materials in the jars with the lowest rates, but did not affect results with the higher rates since the materials were present usually in such excess that liquid or solid material remained unvolatilized on the bottom of each jar at the end of the experiment.

These three experiments, summarized in table 11, showed sharp differences in the ability of the various materials placed in the mason jars to pass through the air and through the cotton plugs and then kill the bits of *Phymatotrichum* sclerotial masses. Pentachlorethane, tetrachlorethane, xylene, and ammonia were completely toxic, at the lowest concentration tested, and with a period of exposure of only 24 hours. Carbon disulphide and a considerable group of other materials were somewhat less effective, and naphthalene and alpha-naphthol were completely ineffective. The two organic mercury preparations included in the tests were ineffective even in the highest concentration with only 1 day of exposure, but toxic to the lowest concentration after 4 days' exposure.

To obviate uncertainty of concentration of materials present in the jars after the first period of exposure, a series of experiments E–5, E–7, and E–8 was run in which a separate mason jar was used as the container for each set of tubes for each harvest. In experiment E–5, ethyl chloride, pentachlorethane, and DuBay 1153–A were included,

the same three weights of materials being used as in the previous experiments. The ethyl chloride was measured as a liquid from a flask cooled in a freezing mixture, and sealed in the jars with little loss. It was not toxic even at the highest concentration after 7 days (table 12). Pentachlorethane killed all the inoculum, at every concentration, within 24 hours. DuBay 1153–A was again completely ineffective even at the highest concentration with only 24 hours' exposure, but prevented all growth from sclerotia that had been exposed to even the lower concentration for 3 or for 7 days.

TABLE 11.—*Toxicity of materials confined in mason jars to sclerotia of Phymatotrichum omnivorum exposed on wads of moist cotton for different periods and then transferred to agar slants*

Experiment number and material tested	Growth (+) or lack of growth (−) of the fungus after exposure for time shown to the indicated weights of material per 2-quart jars								
	0.144 g			0.72 g			2.88 g		
	1 day	3 days	7 days	1 day	3 days	7 days	1 day	3 days	7 days
Experiment E–2:									
Pentachlorethane	−	−	−	−	−	−	−	−	−
Xylene	−	−	−	−	−	−	−	−	−
Ammonia	−	−	−	−	−	−	−	−	−
Carbon disulphide	+	[1] +	−	−	−	−	−	−	−
Perchlorethylene	+	+	+	−	−	−	−	−	−
Formaldehyde	+	[1] +	−	+	−	−	[1] +	−	−
Paradichlorobenzene	+	+	−	+	−	−	+	−	−
None (checks)	+	+	+	+	+	+	+	+	+
Experiment E–3:									
Tetrachlorethane	−	−	−	−	−	−	−	−	−
Trichlorethylene	+	+	+	−	−	−	−	−	−
Dichlorethylene	+	+	+	−	−	−	−	−	−
Carbon tetrachloride	+	+	+	−	−	−	−	−	−
Dichlormethane	+	+	+	+	[1] +	[1] +	−	−	−
Benzene	+	+	+	+	+	+	+	+	−
Naphthalene	+	+	+	+	+	+	+	+	+
None (checks)	+	+	+	+	+	+	+	+	+
	1 day	4 days	7 days	1 day	4 days	7 days	1 day	4 days	7 days
Experiment E–4:									
Chloroform	+	+	[1] +	−	−	−	−	−	−
Ethylene dichloride	+	+	+	−	−	−	−	−	−
Ether	+	+	+	+	+	+	−	−	−
New Improved Ceresan	+	−	−	+	−	−	+	−	−
DuBay 1153–A	+	−	−	+	−	−	+	−	−
Acetone	+	+	+	+	+	+	[1] +	−	−
Alpha-naphthol	+	+	+	+	+	+	+	+	+
None (checks)	+	+	+	+	+	+	+	+	+

[1] Growth from only 1 of 3 slants.

Experiment E–7 was run with pentachlorethane and hexachlorethane, each at additional concentrations of 0.072 and 0.36 g per 2-quart jar. (On the theoretical comparative basis of the weight required as additions to sufficient Houston soil to fill the jars, these would correspond to additions of 50 and 250 p. p. m. of air-dry soil weight.) Pentachlorethane killed all the inocula exposed to any of these concentrations for either 16, 22, or 40 hours. Hexachlorethane, on the other hand, was not toxic at any of the concentrations, after even the longest periods. This solid, most highly chlorinated of the ethane series is thus apparently of no value as a fungicide against *Phymatotrichum omnivorum*.

TABLE 12.—*Toxicity of volatile materials confined in mason jars to sclerotia of Phymatotrichum omnivorum exposed on wads of moist cotton for the periods indicated and then transferred to agar slants, quadruplicate tubes with separate mason jar being used for each period of exposure with each concentration of each material*

Experiment No. and material tested	Periods of exposure	Growth (+) or lack of growth (−) of the fungus after exposure to the indicated weights of material per 2-quart jar				
		0.072 g	0.144 g	0.36 g	0.72 g	2.88 g
Experiment E–5:						
Pentachlorethane	1 day		−		−	−
	3 days		−		−	−
	7 days		−		−	−
DuBay 1153-A	1 day		+		+	+
	3 days		−		−	−
	7 days		−		−	−
Ethyl chloride	1 day		+		+	+
	3 days		+		+	+
	7 days		+		+	+
None (checks)	1 day		+		+	+
	3 days		+		+	+
	7 days		+		+	+
Experiment E–7:						
Pentachlorethane	16 hours	−	−	−	−	
	22 hours	−	−	−	−	
	40 hours	−	−	−	−	
Hexachlorethane	16 hours	+	+	+	+	
	22 hours	+	+	+	+	
	40 hours	+	+	+	+	
None (checks)	16 hours	+	+	+	+	
	22 hours	+	+	+	+	
	40 hours	+	+	+	+	
Experiment E–8:						
Tetrachlorethane	16 hours	−	−	−	−	
	24 hours	−	−	−	−	
	3 days	−	−	−	−	
Xylol (industrial)	16 hours	+	−	−	−	
	24 hours	+	−	−	−	
	3 days	+	−	−	−	
Gasoline (industrial)	16 hours	+	+		+	+
	24 hours	+	+		+	[1] Tr.
	3 days	+	+		−	−
	7 days	+	+		−	−
None (checks)	16 hours	+	+	+	+	+
	24 hours	+	+	+	+	+
	3 days	+	+	+	+	+
	7 days	+	+	+	+	+

[1] Growth from only 1 slant.

Experiment E–8 (table 12) was a comparison of tetrachlorethane with industrial xylol and industrial gasoline. In the series were included the short period of exposure and low rate of application as used in the previous experiment. Tetrachlorethane was completely effective in this stringent test, while xylol was not effective at the lowest rate with any of the periods of exposure and gasoline was effective only after 3 days exposure to the higher rates. Use in these experiments of the shorter period of exposure and lower rates permitted differentiation of pentachlorethane and tetrachlorethane as slightly more toxic than xylol under these conditions.

COMPARISON OF TOXICITY RATINGS WITH THE FUNGISTATIC EFFECTIVENESS OF MATERIALS AFTER PENETRATING THE SOIL

In the experiments recorded above some possibly fungicidal materials have been considered chiefly from two aspects. The studies in soil chambers (tables 1 to 9) were essentially fungistatic tests, designed

primarily to determine the ability of the materials at various concentrations to penetrate moist soil and there to inhibit growth of the root rot fungus. In the toxicity tests (tables 10 to 12), the materials were judged by another criterion, namely, their ability to volatilize through air and through dry cotton plugs and then kill or permanently inactivate small portions of *Phymatotrichum* sclerotial masses. Obviously, materials highly effective by both tests are more promising as possible fungicides than materials that proved to be effective by one but not by both tests. It is thus of interest to compare the results in summarized form, as in table 13.

TABLE 13.—*Comparative effectiveness of materials in killing sclerotial masses of Phymatotrichum omnivorum exposed only to fumes of the materials, and in penetrating moist soil to inhibit growth from sclerotial masses*

[Arranged with the more effective materials at the top]

Toxicity rating [1]		Fungistatic rating [2]	
Conditions	Material	Rates of application	Material
		Parts per million	
1 day, at low concentration.	Pentachlorethane. Tetrachlorethane. Xylene. Ammonia.		Pentachlorethane. Tetrachlorethane. Xylene. Carbon disulphide.
1 day at medium or 4 days at low concentration.	Carbon disulphide. Perchlorethylene. Trichlorethylene. Dichlorethylene. Carbon tetrachloride. Chloroform. Ethylene dichloride. New Improved Ceresan. DuBay 1153–A.	100	Perchlorethylene. Trichlorethylene. Dichlorethylene. Turpentine. Paradichlorobenzene. Chloroform. Dichlormethane. Benzene. Gasoline.
1 day at high or 7 days at low concentration.	Formaldehyde. Paradichlorobenzene. Dichlormethane. Ether.	500 1,000 2,000	Kerosene. Ethyl chloride. Ether. Naphthalene.
3, 4, or 7 days at high concentration.	Gasoline. Benzene. Acetone.		New Improved Ceresan. DuBay 1153–A. DuBay 971–A. Semesan.
Not toxic in limits of tests.	Ethyl chloride. Hexachlorethane. Naphthalene. Turpentine. Alpha-naphthol.	[3] 2,000	Acetone. Cresylic acid. Sulphur chloride. Hexamethylenetetramine. Alpha-naphthol. Petroleum (lubricating) oil.
		[3] 4,000	Formaldehyde.
		[3] 10,000	Ammonia.

[1] Volatilizes through air and cotton plugs, and kills sclerotial masses in time indicated.
[2] Penetrates 135 mm of moist soil and inhibits growth when applied in soil chambers at rates indicated.
[3] Not effective at concentration given.

Three of the materials, pentachlorethane, tetrachlorethane, and xylene, were completely effective in low concentrations by both criteria. Another group of materials, including carbon disulphide, perchlorethylene, trichlorethylene, and dichlorethylene, were effective at 100 p. p. m. in the test of fungistatic effect, but fell into the second group in the toxicity test. These materials are, therefore, apparently not quite so likely to be effective under soil conditions as the first three. Paradichlorobenzene, which belongs in the first group on the basis of its ability to penetrate soil and inhibit growth, is shown by the toxicity test to be rather slowly toxic.

Some of the other materials appeared more promising from the standpoint of toxicity than from that of ability to penetrate the soil and inhibit growth. Thus New Improved Ceresan and DuBay 1153–A were fairly toxic but were not able to penetrate the soil to any practical extent. Ammonia was very toxic but did not penetrate the soil effectively even when added at 10,000 p. p. m., the highest rate tested for any of the materials. Formaldehyde was moderately toxic, but of no apparent value when judged by its ability to penetrate moist Houston black clay soil.

Laboratory study of a variety of materials by these several tests has furnished rather critical data for determining their probable value as fungicides against *Phymatotrichum* root rot. While the tests are completely arbitrary, materials that fail to be effective in these laboratory tests will probably be less useful under field conditions than materials that are effective in the tests. The results indicate that the general failure of some fungicides to eradicate *Phymatotrichum* root rot is due probably to their low effectiveness in permeating the soil.

On the basis of the results obtained, pentachlorethane, tetrachlorethane, and xylene appear to be particularly worthy of field trial against root rot. Obviously, however, none of these materials should be recommended for practical use without thorough trial in field experiments. In field trials against *Phymatotrichum* root rot, pentachlorethane, tetrachlorethane, and xylol applied around infected plants have regularly killed the fungus in the roots of the plants. As yet, however, the methods tried for applying these materials in the field have not succeeded in completely eradicating the disease from infested areas. These field experiments are summarized in an accompanying paper (*3*). None of the soil fungicides mentioned in the present paper are recommended at this time for practical use against *Phymatotrichum* root rot.

SUMMARY

Methods were developed for evaluating fungicides in the laboratory as to ability to permeate soil and as to fungistatic and fungicidal effectiveness against the root rot fungus, *Phymatotrichum omnivorum*.

Tests with fungicides mixed mechanically into soil indicated the relative effectiveness of a number of organic mercury compounds for preventing growth from inoculum of *P. omnivorum*. The initial fungistatic effectiveness of ethyl mercury materials was found to be much higher than would be expected from their mercury content, but the residual value after 5 weeks' contact with moist soil was in line with that of other compounds.

Tests with fungicides applied on the surface of Houston black clay soil in closed jars showed whether materials were able to penetrate moist soil in jars, to various depths, and then prevent growth of *P. omnivorum*. A group of volatile materials, including pentachlorethane, tetrachlorethane, xylene, carbon disulphide, turpentine, perchlorethylene, trichlorethylene, and dichlorethylene, applied at only 100 p. p. m. of the air-dry soil weight, completely inhibited growth from inoculum 135 mm deep in the soil. Formaldehyde, on the other hand, was ineffective even at 4,000 p. p. m.; and ammonia did not prevent growth from deep inoculum even at 10,000 p. p. m. The organic mercury compounds tested prevented growth from inoculum near the surface but not from deeper inoculum.

By using a method in which the inoculum was inserted deep in soil in closed jars several weeks after pentachlorethane had been applied on the surface, it was found that 4 to 8 weeks' contact with moist soil reduced the fungistatic effectiveness to between a fifth and a tenth of the original value.

Pentachlorethane, tetrachlorethane, and xylene when applied at only 100 p. p. m. on the surface of Houston soil in closed jars were quite effective in preventing growth from *P. omnivorum* inoculum buried in the soil, but these low concentrations were not regularly effective under more adverse conditions, as with very compact soil, or with infected roots as the inoculum, or in experiments in which the jars were left open and fanned to accelerate surface loss of volatile fungicides. Higher concentrations—500 to 1,000 p. p. m.—appeared necessary under these conditions. In open jars, there appeared considerable advantage in inserting the fungicide in holes below the surface rather than applying it on the surface; yet in one experiment the inoculum located just below the surface of the soil survived such treatment.

Actual toxicity of the volatile materials was determined by a method that required the fungicides to volatilize through air and through cotton plugs before coming in contact with the fungus inoculum. Pentachlorethane, tetrachlorethane, xylene, and ammonia proved most toxic to *P. omnivorum* in these tests.

Pentachlorethane, tetrachlorethane, and xylene showed ability to penetrate moist soil and prevent growth of *P. omnivorum*, together with high fungicidal value after passage through air, and these materials are suggested as promising soil fungicides for trial in field experiments. None of the soil fungicides mentioned in this paper are recommended at this time for practical use against *Phymatotrichum* root rot.

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TESTS WITH PENTACHLORETHANE, TETRACHLORETHANE, AND XYLOL TO DETERMINE THEIR EFFICIENCY IN ERADICATION OF PHYMATOTRICHUM ROOT ROT[1]

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INTRODUCTION

An accompanying paper (*1*) [2] summarizes previous studies on possible control by soil fungicides of root rot caused by *Phymatotrichum omnivorum* (Shear) Duggar, and presents results of laboratory comparisons of chemicals for their relative value as soil fungicides against *P. omnivorum*. These experiments demonstrated that most of the water-soluble materials tested, such as formaldehyde, ammonia, and organic mercury compounds, were apparently unable to penetrate rapidly through moist Houston black clay soil under the conditions of the tests. A number of other materials, including pentachlorethane, tetrachlorethane, and xylene, mostly not water-soluble, readily permeated the soil. Separate comparisons of the relative toxicity of chemicals to *P. omnivorum*, under conditions that required passage through air and dry cotton plugs, indicated high toxicity values also for pentachlorethane, tetrachlorethane, and xylene. These results suggested the desirability of field trial of the three chemicals for possible use against *Phymatotrichum* root rot. The present paper summarizes the trials made with these materials between September 1933, and October 1935.

MATERIALS AND METHODS

The materials used were commercial refined pentachlorethane,[3] specific gravity 1.685, commercial refined tetrachlorethane, specific gravity 1.58, and industrial xylol (xylene), specific gravity 0.86. Some experiments included also a partially refined tetrachlorethane, referred to below as "crude" tetrachlorethane. This was a somewhat turbid, dark greenish-yellow liquid with a pungent odor.

The experiments were carried out at College Station and Bryan, Tex., with various plants growing in Lufkin fine sandy loam soil. This soil has a highly plastic and impervious clay subsoil; nevertheless the roots of cotton and other plants penetrate it deeply.

The experiments were of three distinct types, and the special methods used will be presented separately. However, the fungicides were applied in each case in holes pierced with a crowbar to a depth of 6 inches. One such hole was used for each 6 inches square of the area to be treated, or four holes to each square foot. The holes were located by measurement uniformly over treated areas.

[1] Received for publication July 14, 1937; issued May 1938. Contribution No 408, Technical Series, Texas Agricultural Experiment Station.

[2] Reference is made by number (italic) to Literature Cited, p. 593.

[3] The pentachlorethane and tetrachlorethane used in these experiments were furnished through the courtesy of the R. & H. Chemicals Department, E. I. du Pont de Nemours & Co.

The laboratory results (*1*) had indicated that while fungicide concentrations of 100 parts per million of the soil weight were effective in closed jars, concentrations of 500 to 1,000 parts per million would probably be necessary for effective use under more or less adverse conditions. Accordingly, the following rates were calculated:

Rates of application, as grams per square foot	*Equivalent to concentrations as parts per million of soil weight to a depth of 4 feet*
249	1,500
166	1,000
83	500
41.5	250

It should be emphasized that while the theoretical depth of 4 feet was arbitrarily used in calculating rates of application, the fungicides were actually applied only in the holes 6 inches deep, and not to the depth mentioned. Weights of fungicides for each hole were calculated to volume. These volumes were measured in the field for each hole, the material was poured in, and the soil pushed into the top of the hole. Each hole was covered in this way with soil rapidly enough to avoid appreciable evaporation of the volatile materials from the open holes; but the materials were found to evaporate later through the soil. Odors of the various fungicides were noticed over treated plots for several days or even weeks after the materials had been applied.

The first field trials of these volatile fungicides were made by applying them in relatively small areas surrounding plants recently attacked by root rot, and then after various periods digging out the roots of the plants and determining the viability of the fungus on and within the roots. The roots were obtained by digging a trench near the plants, and then separating the roots of each plant from the soil by carefully picking the remaining soil away with ice picks. The roots were cut at once into sections corresponding to the depths in the soil, and placed in mason jars containing a little water. These jars were taken into the laboratory, the excess water was poured out, and Houston black clay surface soil containing 25 percent moisture on an air-dry soil-weight basis was filled in around the roots. Soil cultures prepared in this way are a more reliable means of demonstrating the viability of *Phymatotrichum omnivorum* on diseased roots than are cultures prepared aseptically on culture media (*6, p. 771*). The fungus if alive grows out from the root and develops in the soil-glass interface where it is readily observed. In the present experiments, soil cultures were observed usually for 4 to 6 weeks, and doubtful growth was always examined under the microscope.

Absence of growth in soil cultures made in this way from treated roots was taken to indicate that the fungus was not viable. However, two possible sources of error should be noted. (1) A root from a treated area might carry with it fungicide taken up from the soil and inhibit growth from fungus material not actually killed. (2) An area selected for experimental treatment might be one in which plants had been attacked by the disease some time before. As has been pointed out (*6, pp. 768–773*), *Phymatotrichum omnivorum* has never been recovered by the writer or his associates from thoroughly decayed roots of plants that have succumbed to the disease, and failure to obtain the fungus from fungicide-treated roots of this kind could not be considered evidence that the fungicide had killed the fungus. To obviate this

difficulty, these experiments were invariably carried out with plants only recently attacked, on the roots of which the fungus would still be advancing actively, and untreated, check areas were always selected with the disease in comparable stages.

RESULTS OF APPLYING THE MATERIALS AROUND DISEASED PLANTS

APPLICATION OF TETRACHLORETHANE AROUND COTTON PLANTS IN 1933

A preliminary field test with tetrachlorethane was run in a cotton (*Gossypium hirsutum* L.) field at College Station during the fall of 1933. Ten comparable areas were selected, on September 21, along the advancing margins of root rot spots. Tetrachlorethane was applied in areas of 12 square feet, the treated areas extending 4 feet along the row and 1½ feet to each side. Each area included several plants that had just succumbed to root rot and several others that as yet showed no signs of the disease above ground, but were presumably already infected. Three areas in different parts of the field were marked off and treated at 166 g and three at 83 g per square foot, and four additional areas were held as checks.

Close observation during the experiment showed no definite injury to the treated plants that could be attributed to the tetrachlorethane. Plants in adjoining rows, only 1½ feet from the treated areas, showed no injury from the tetrachlorethane during the several months before frost.

The effect of the treatment on the root rot fungus was determined, after 15, 21, and 32 days, by excavating sample areas of each rate of application and the checks. Trenches 2 feet deep were dug next to the plants, and the roots were separated and placed in soil cultures for testing as described above. The results are summarized in table 1.

TABLE 1.—*Effects of tetrachlorethane applications at different rates around cotton plants with root rot, September 1933*

Rate of application per square foot (grams)	Interval before excavation of roots	Plants in plot	Results of testing portions of roots in jars of moist soil		
			Portions tested	Portions yielding *Phymatotrichum omnivorum*	
	Days	*Number*	*Number*	*Number*	*Percent*
166	15	8	20	0	0
	21	5	15	0	0
	32	5	15	0	0
83	15	7	18	1	6
	21	7	21	0	0
	32	7	23	0	0
0 (checks)	15	7	25	15	60
	21	4	15	4	27
	32	6	14	2	14
	32	5	12	1	8

Profuse *Phymatotrichum* strand growth developed from the roots of at least one plant from each check area. From the 39 cotton plants from the treated areas, on the contrary, there was growth only from the 12- to 18-inch deep portion of one taproot, in exceptionally dense clay, from a plot treated at the lower rate and dug after 15 days. As

will be noted in figure 1, this root was located near the edge of the treated area.

Figure 1 shows diagrammatically the results obtained from the

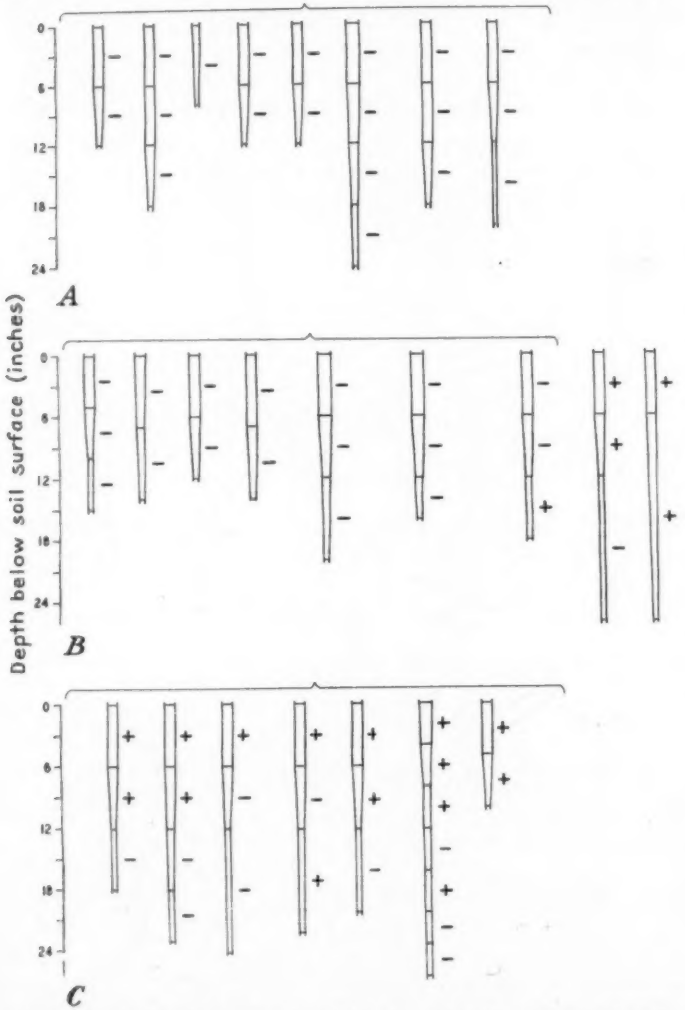

FIGURE 1.—Diagrammatic summary of results in plots excavated 15 days after tetrachlorethane was applied in areas designated by braces. Cotton plants were located within or near the plots at approximate positions shown. Roots were cut into portions as indicated, later growth of *Phymatotrichum* strands from these portions in soil cultures being indicated by + and lack of growth by −: *A*, Application at rate of 166 g per square foot; *B*, application at rate of 83 g per square foot; *C*, not treated, check.

three plots excavated 15 days after tetrachlorethane had been applied. It will be noted that the root rot fungus had been killed by this time on roots from the treated area, with the exception mentioned, but

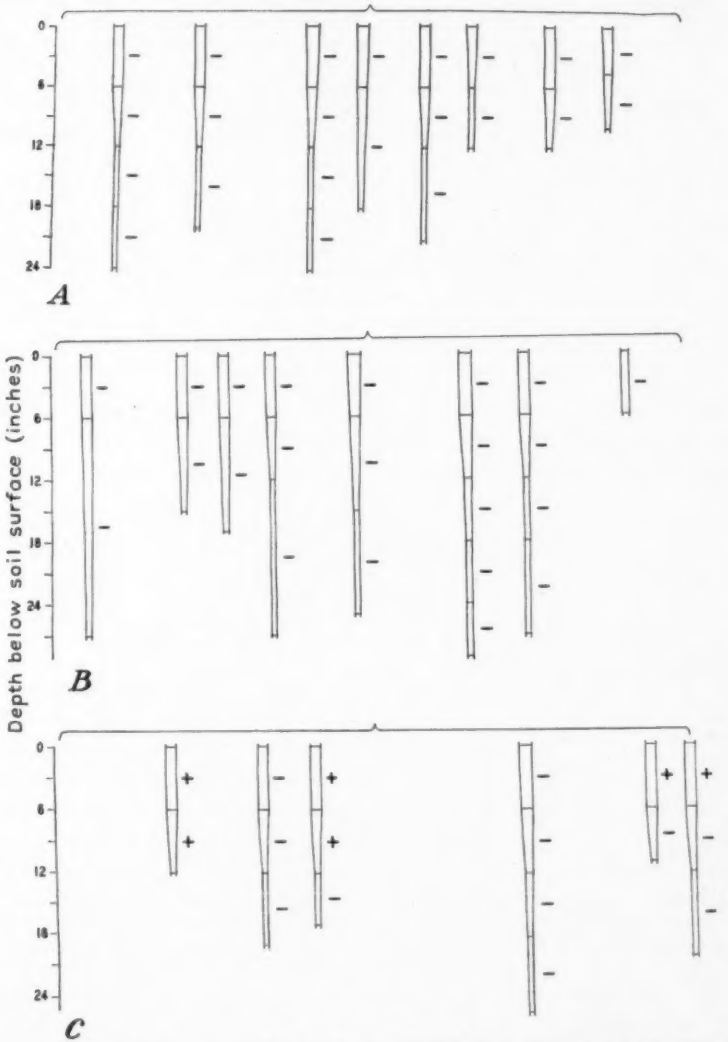

FIGURE 2.—Diagrammatic summary of results in plots excavated 34 to 39 days after industrial xylol was applied in areas designated by braces. Cotton plants were located within the plots at approximate positions shown. Roots were cut into portions as indicated, later growth of *Phymatotrichum* strands from these portions in soil cultures being indicated by + and lack of growth by −: *A*, application at rate of 166 g per square foot; *B*, application at rate of 83 g per square foot; *C*, application at rate of 41.5 g per square foot.

that it was still alive on portions of roots from the untreated check, and on roots from plants immediately adjoining the treated area. Growth of *Phymatotrichum* resulted also from portions of roots from adjoining rows, only 1½ feet distant from the side of the treated areas. Similar additional tests with roots of plants from outside the treated areas were made on each of the three dates of harvest, and it was found uniformly that very little if any toxic effect had occurred more than a few inches horizontally beyond the areas in which the tetrachlorethane had been applied.

Downward permeation of the tetrachlorethane through the soil was evidenced by direct observation of the odor while the roots were being dug, as well as by the indirect evidence of the effect on the fungus. In at least one instance, the tetrachlorethane odor was strong in soil at a depth of 28 inches, after 3 weeks. The odor was still noticeable in the soil at the end of the experiment, after 32 days.

In this experiment, tetrachlorethane applied at 166 g per square foot, in holes only 6 inches deep, was successful in killing or at least permanently inhibiting later growth of the fungus on or within affected cotton roots down to a depth of at least 2 feet. Application in four holes per square foot was evidently sufficient for reasonably uniform permeation despite the dense subsoil.

APPLICATION OF TETRACHLORETHANE AND XYLOL AROUND COTTON PLANTS, IN 1934

A more extensive series of tests was run in the fall of 1934 in the same cotton field at College Station. On August 29 and 30, 24 comparable areas were marked off on the advancing edges of root rot spots in this field. As before, each plot was carefully located to include some plants already showing signs of root rot and others as yet without above-ground symptoms. Each plot extended 5 feet along the row and 2 feet to each side. Applications were made on 18 plots, the remaining 6 serving as checks. Duplicate plots were used, the applications being at the rate of 166, 83, and 41.5 g per square foot for industrial xylol, commercial refined tetrachlorethane, and crude tetrachlorethane. Roots from half the plots were excavated after 15 to 19 days, and from the other plots after 34 to 40 days. These plots were excavated usually to depths of 3 feet or more, but few roots were recovered at greater depths than 24 to 28 inches (fig. 2).

The results of this experiment are summarized in table 2. At 166 g per square foot perfect control was obtained on deep and shallow portions of the roots with all three fungicides. At 83 g per square foot, the results were similar except that *Phymatotrichum* growth was obtained from 1 of the 112 portions of roots tested. At the lowest concentration, 41.5 g per square foot, the fungus remained alive in one root exposed to crude tetrachlorethane and in several roots exposed to xylol (fig. 2), and grew from 7 of the 79 portions tested. In the accompanying tests of untreated check plants, the fungus grew from 40 of the 111 root portions tested.

The roots excavated after 39 days from the plot treated with xylol at 41.5 g per square foot yielded a higher percentage of living *Phymatotrichum* than did roots from untreated plots excavated after the same interval. This is possibly to be explained as follows: In check plots, the viability of the fungus decreased in general with time (tables 1

and 2) as a result of gradual decay of roots and subsequent death of the fungus. But in the plot mentioned, continued decay of roots presumably was held in check by the xylol, which, however, was not maintained at a concentration sufficient to kill the fungus. With gradual dissipation of the xylol during the 39-day interval, the fungus was therefore still viable. Similar apparently favorable effects of low concentrations of fungicides have sometimes been found in laboratory tests with organic mercuries, which at concentrations too low to impede the growth of *Phymatotrichum omnivorum* frequently encourage more extensive growth than in the accompanying untreated checks.

TABLE 2.—*Effects of applying refined and crude tetrachlorethane and industrial xylol at different rates around cotton plants with root rot, August 1934*

Fungicide	Rate of application per square foot	Interval before excavation of roots	Plants in plot	Results of testing portions of roots in jars of moist soil		
				Portions tested	Portions yielding *Phymatotrichum omnivorum*	
	Grams	*Days*	*Number*	*Number*	*Number*	*Percent*
Tetrachlorethane	166	15	8	16	0	0
		34	9	26	0	0
	83	16	11	23	0	0
		35	8	17	0	0
	41.5	19	5	10	0	0
		39	7	19	0	0
Tetrachlorethane, crude	166	16	8	16	0	0
		34	8	21	0	0
	83	16	6	10	0	0
		36	9	28	1	4
	41.5	19	5	7	1	14
		40	10	15	0	0
Xylol, industrial	166	15	7	15	0	0
		34	8	22	0	0
	83	16	7	12	0	0
		34	8	22	0	0
	41.5	19	6	11	0	0
		39	6	17	6	35
None (checks)		16	5	10	2	20
		17	8	22	19	86
		19	9	16	12	75
		34	9	27	4	15
		39	9	21	2	10
		39	8	15	1	7

In general, these results agreed with those obtained in laboratory studies with materials applied on the surface of soil in open jars (*1, table 8*). Under these conditions, a concentration of 500 parts per million proved too low for inhibition of growth of the fungus, while higher concentrations were effective. Similarly, under the conditions of the present experiment, applications of between 83 and 166 g per square foot of any of the fungicides used will probably be necessary. The present experiment confirmed the test of the previous year in indicating that treatments of this kind could kill the root rot fungus in and on cotton roots well below the depth at which the fungicides were applied.

APPLICATION OF PENTACHLORETHANE AROUND A CHINABERRY TREE, IN JULY 1934

A 10-year-old chinaberry (*Melia azedarach* L.) tree, just beginning to wilt from an attack of root rot, was used for the following experi-

ment. An area 8 by 16 feet at one side of the tree was treated with pentachlorethane at the rate of 166 g per square foot. The soil at the other side of the tree was left untreated. Plots approximately 5 by 5 feet were excavated after 3 and 6 weeks from the treated and from the check areas. The roots within the plots were removed to a depth of about 5 feet, sawed into convenient portions, and placed as usual in soil cultures.

A condensed summary of the results is given in table 3. Of the roots from the treated area, only one portion one-half inch in diameter, taken after 3 weeks from a depth of 33 to 39 inches, yielded viable *Phymatotrichum*. Other infected roots of various diameters up to 2 inches and located to a depth of 5 feet in the treated area failed to yield growth of the fungus. This is of less significance, however, since the deeper roots from the untreated, check area also failed to yield growth, the growth recorded coming from roots at a depth of 12 to 18 inches.

APPLICATION OF PENTACHLORETHANE AROUND AN ELM TREE IN AUGUST 1934

A 9-year-old elm (*Ulmus pumila* L.) tree just showing signs of root rot was used in this experiment. An area 8 by 16 feet at one side of the tree was treated with pentachlorethane at the rate of 83 g per square foot. Roots were excavated after 31 days from a 6- by 6-foot plot within this treated area and from a similar plot on the other side of the tree. The results are given in table 3.

TABLE 3.—*Results of applying pentachlorethane at the rate of 166 g per square foot around a chinaberry tree and of 83 g per square foot around an elm tree, both of which were affected with root rot.*

Tree and location of plots	Interval before excavation of roots	Results of testing of portions of roots in jars of moist soil		
		Portions tested	Portions yielding *Phymatotrichum omnivorum*	
	Days	*Number*	*Number*	*Percent*
Chinaberry:				
Treated area	21	54	1	2
	42	33	0	0
Untreated area (check)	23	41	2	5
	43	33	6	18
Elm:				
Treated area	31	36	0	0
Untreated area (check)	31	42	22	52

The roots from the treated area, within which the fungus apparently was killed by the relatively light treatment, varied from ¼ inch to 2 inches in diameter, and were found at depths of 4 to 20 inches only, although the excavation was continued to about 5 feet. Strand growth from the untreated roots was from roots ⅛ to 3 inches in diameter, found at depths of 4 to 12 inches only. *Phymatotrichum* on the shallow roots of this elm tree was killed by application of pentachlorethane at 83 g per square foot.

ATTEMPTED ERADICATION OF ROOT ROT WITH TETRACHLORETHANE AND XYLOL

While the experiments summarized above indicated that the fungicides applied at 83 or 166 g per square foot would kill the root rot fungus on roots within the treated areas, they did not indicate whether such treatment would actually eradicate the fungus from infested areas. *Phymatotrichum* may survive on deeper roots than those recovered for testing, and it may survive also as sclerotia at various depths in the soil. The following experiment was therefore planned as a rather crucial test of the possibility of eradicating root rot by applying the fungicides by the method used in the other tests—that is, in holes punched to a depth of 6 inches.

The applications were made in plots bordered by "sorghum barriers." This precaution had not previously been used in experiments

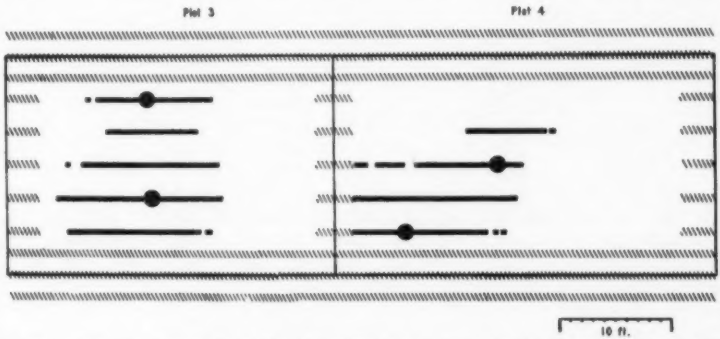

FIGURE 3.—Diagram of two plots used in the eradication experiment, showing extent of root rot in 1935. Continuous lines show edges of areas treated in 1934 with xylol and tetrachlorethane, respectively, and hachured lines show location of sorghum barrier rows. Dots indicate centers where root rot first recurred on July 26, 1935, and heavy lines the extent of root rot spread from these points by October 29, 1935.

on the eradication or control of *Phymatotrichum* root rot, and the value of such experiments had been weakened by the possibility that areas from which the fungus had been nearly or quite eradicated might periodically be reinfested from adjoining untreated areas. When cotton plants are used, this source of error can now be eliminated since it has been shown (*7*) that a few intervening rows of sorghum plants will act as a barrier to prevent spread of root rot from infected cotton plants into an immediately adjoining uninfested plot, over a period of years. A plot completely enclosed by rows of sorghum plants should, therefore, be safe from the spread of root rot from neighboring plots. This being so, it is possible to determine accurately the immediate or cumulative effect of attempted eradication or control methods. In the present experiment, the sorghum barriers along the sides of the plots consisted of three rows 2 feet apart. The ends of the plots were protected by planting sorghum for 3 feet at each end of each row of cotton. Each plot was thus enclosed completely by the sorghum border without interfering with cultivation (fig. 3).

TABLE 4.—*Synopsis of root rot eradication experiment by the use of xylol and tetrachlorethane in field plots of cotton guarded by sorghum barriers,*[1] *1934*

Plot No.	Fungicide, applied at 249 g per square foot	Time and conditions of application	Centers of infection appearing after the treatment	Results in 1935		
				Plants	Plants with root rot on—	
					Aug. 20	Oct. 29
			Number	*Number*	*Percent*	*Percent*
1	Xylol	Apr. 18–23, in wet soil	1	281	1.8	28.1
2	Tetrachlorethane	Apr. 2–9, in wet soil	3	336	0	13.4
3	Xylol	Aug. 9–11, in dry soil	2	295	4.4	47.5
4	Tetrachlorethane	Aug. 7–8, in dry soil	2	300	4.0	37.0
5	None (check)		(2)	523	99.8	100.0

[1] Plots 1 and 3 were each 20 by 30 feet in size; plots 2 and 4, 20 by 34 feet; and plot 5, 20 by 64 feet.
[2] About 55.

The experiment was located on plots that had served as check areas in a previous experiment. Root rot was introduced here by inoculation in 1929, and had become increasingly prevalent on cotton grown year after year, attacking nearly 100 percent of the plants in 1931, 1932, and 1933. The general plan of the present experiment included treatments with xylol and tetrachlorethane at the high rate of 249 g per square foot in wet soil in spring and in dry soil in summer (table 4). The applications in plots 1 and 2 were made before the crop was planted, in soil so wet that the fungicides were absorbed quite slowly from the holes into which they were poured. The applications in August were made after a long drought, and the fungicides were absorbed rapidly into the soil.

INJURY FROM THE FUNGICIDES

Cotton and sorghum were planted in these plots a month after the tetrachlorethane was applied in plot 2, and only 2 weeks after xylol was applied in plot 1. A good stand, with relatively little retardation of growth, was obtained in plot 2, but after repeated planting only occasional stunted plants were obtained in plot 1. In plots 3 and 4 the fungicides were applied around the growing cotton plants and caused severe injury. Within 1 month the plants in plot 4 were dead, and a month later all the plants in plot 3 had succumbed.

On March 7, 1935, small chinaberry trees were planted in each treated plot, and these as well as the cotton, planted April 1, showed residual injurious effects of the treatments. By June 29 the condition of the plants was as follows:

Plot 1, cotton plants 60 to 110 cm tall, chinaberry trees all alive.
Plot 2, cotton plants 15 to 25 cm tall, chinaberry trees all dead.
Plot 3, cotton plants 80 to 110 cm tall, chinaberry trees all alive.
Plot 4, cotton plants 60 to 95 cm tall, chinaberry trees 9 of 12 alive.

A full year after the tetrachlorethane was applied in the wet soil in plot 2, there was still severe injury, while the same material applied only 8 months previously in dry soil caused less injury in plot 4. Neither of the xylol-treated plots showed definite injury from the fungicide in 1935. Xylol was thus much less injurious than tetrachlorethane both in immediate and residual effects.

RECURRENCE OF ROOT ROT

Following the fungicide applications in 1934, root rot was observed only in plot 1. It appeared in October in a single center, and by December 10 had involved 6 of the 343 plants in the plot.

In 1935, the treated plots were still free of above-ground evidence of root rot until the end of June. By that time more than 20 percent of the plants in the check plot had been killed by the disease, which appeared at numerous points along each row. Root rot was observed in the treated plots on the following dates: Plot 1, July 6; plot 2, September 19; plot 3, July 26; and plot 4, July 26. It spread rapidly from only a few centers in each plot, and had involved a total of 30 plants by August 20, when all but one of the plants in the check plot had succumbed to the disease. However, the need of complete eradication to make fungicide treatment of value was well illustrated by the continued spread of root rot after this date over a large portion of the treated plots (table 4). Of 29 chinaberry trees that survived injury from the fungicides, 10 were killed by root rot in 1935.

The incidence of root rot in these treated plots was delayed and the prevalence of the disease reduced during the season following treatment, but the treatment failed to eradicate root rot. Possible causes of this failure are discussed below.

EFFECTS OF THE FUNGICIDES ON HOST PLANTS

Most of the experiments to determine the fungicidal value of the materials furnished little evidence as to their effect on the host plants, since the plants were dug out generally within a few weeks after treatment. Data were therefore obtained on this point by special treatments around some trees and shrubs growing in an experimental nursery at College Station, in small plots extending 10 feet along the rows and 2 feet to each side. During the summer of 1934, fungicides were applied within 20 such areas at 166, 83, and 41.5 g per square foot.

Of the three fungicides, tetrachlorethane was rapidly and severely injurious, xylol next, and pentachlorethane was least injurious. Crude tetrachlorethane was approximately as injurious as the refined tetrachlorethane, in a few tests. Rows of pomegranate (*Punica granatum* L.) bushes were treated at 166 g per square foot early in May. The tops of about 90 percent of those treated with pentachlorethane were apparently uninjured 6 months later, whereas most of those treated with tetrachlorethane were dead. Hackberry (*Celtis laevigata* Willd.) trees treated in May with tetrachlorethane and xylol at 166 g succumbed during July and August, respectively, while trees treated at the same rate with pentachlorethane were still apparently uninjured a year later. Similar results were obtained with some other plants.

The final effects of the various treatments are summarized in table 5. It will be noted that hackberry and retama (*Parkinsonia aculeata* L.) trees, and most of the pomegranate bushes, survived the highest rate of treatment with pentachlorethane without definite injury. At the same rate, the tetrachlorethane and xylol treatments killed hackberry trees and more than half of the pomegranate bushes, only the

retama proving resistant. In descending order of susceptibility to fungicide injury, the plants ranked approximately as follows:

Live oak (*Quercus virginiana* Miller), elm, ilex, hawthorn (*Crataegus*), hackberry, pomegranate, and retama.

TABLE 5.—*Summary of injurious effects produced within 1 year by applications of volatile fungicides in soil around woody plants in nursery rows*

Materials and rates applied (grams per square foot)	Plants killed	Plants surviving
Tetrachlorethane:		
166	Hackberry; ilex; pomegranate, 75 percent.	Retama; pomegranate, 25 percent.
83	Live oak; pomegranate, 70 percent.	Hackberry; pomegranate, 30 percent.
41.5		Hackberry; pomegranate.
Xylol:		
166	Hackberry; pomegranate, 55 percent.	Retama; pomegranate, 45 percent.
83	Elm; pomegranate, 20 percent.	Hackberry; pomegranate, 80 percent.
41.5		Hackberry, hawthorn, pomegranate.
Pentachlorethane:		
166	Pomegranate, 30 percent.	Hackberry; retama; pomegranate, 70 percent.
83	Pomegranate, 25 percent.	Hackberry; pomegranate, 75 percent.
41.5		Hackberry.

A special comparison was made of xylol applied throughout an area 5 by 5 feet square around a hackberry tree, and in an area similar except that a 2- by 2-foot space was left untreated immediately around the trunk of the tree. The first tree showed yellowed leaves soon after treatment and was dead by the end of the season, whereas the second tree was apparently uninjured a year later.

In general, the three fungicides were injurious to some plants at the two higher concentrations, only the lower rate of 41.5 g per square foot proving generally safe. Since by the method of application used this lower rate was not uniformly effective against the root rot fungus on diseased plants, curative treatment of infected woody plants with these fungicides will probably be impractical. For further experiments, pentachlorethane would be suggested rather than either of the other materials, since of the fungicides tested it was least injurious to the plants and most effective against the fungus. In experiments aimed at eradication of root rot without particular consideration of plants already growing in the infested area, the less expensive [4] (although more injurious) tetrachlorethane or xylol might be given first choice. Tetrachlorethane may be expected to have a much longer residual injurious effect on future plantings than xylol, so xylol might somewhat arbitrarily be suggested as possibly the most promising of the three materials for further consideration in eradication experiments.

DISCUSSION

A chemical eradication method for use against *Phymatotrichum* root rot would be valuable at the present time for experimental and limited practical use only. It would be used, as was pointed out

[4] Pentachlorethane was available at about 15 cents, tetrachlorethane at 8 cents, and industrial xylol at about 4 cents per pound respectively.

earlier (*2*, *5*), chiefly for small root rot spots occurring in otherwise uninfested areas and for emergency eradication should the disease be carried to new areas. Such a method might later be of value also for eliminating final points of infestation in fields where partial eradication had been attained by other and less costly methods. In any case, practical use of fungicides for treatment of the soil would seem to be limited to materials and methods that can effect actual eradication of the fungus, since partial control would scarcely justify the cost of materials and labor. Complete eradication would be particularly desirable when planting susceptible trees and shrubs in previously infested soil, since even scattered infection by *Phymatotrichum* causes rapid loss of orchard and other susceptible perennial plantings (*8*).

The experiments reported above included field tests of pentachlorethane, tetrachlorethane, and xylol, applied always in holes 6 inches deep and covered only by pushing soil into the holes. Treatments around infected plants in small plots, as summarized in tables 1 to 3, indicated that under these conditions applications of 83 to 166 g per square foot regularly killed the fungus on and within roots of the plants down to the depth recovered. Of 253 portions of roots tested from plots treated at the higher rate, the fungus was viable only on 1 portion taken from a depth of 33 to 39 inches. With this exception, infected roots recovered to depths of 2 feet or more did not yield growth of *Phymatotrichum* strands, although the fungicides had been applied in holes only 6 inches deep. These field results thus confirmed the preliminary laboratory results which had suggested that the materials used here were capable of rapidly permeating the soil as well as killing the root rot fungus in approximately the concentrations provided by these applications.

The high cost of chemical treatment of the soil would be justified for therapeutic treatment of valuable diseased trees and shrubs if the fungicides could be used without injury to the host plants in concentrations sufficient to kill the fungus. The applications reported in the present paper suggest that this will probably not be possible, at least by the method of application used in these experiments. In general, applications at 83 or 166 g per square foot injured some of the plants tested. The lower rate, 41.5 g per square foot, did not injure the plants used in these tests, but it failed to kill the fungus in some cases. Curative treatment of infected woody plants with these fungicides will probably be impractical.

The possibility of using tetrachlorethane or xylol for eradicating root rot from infested plots was explored in a separate experiment in which the freedom from root rot of the succeeding crops was used as the criterion rather than the death of the fungus on roots recovered from treated plots. Root rot was markedly diminished in the treated plots, but recurred in each case from a few scattered points. Since the experimental plots were adequately guarded by sorghum barriers, this recurrence of root rot well inside the treated plots was due not to re-encroachment of the fungus from surrounding untreated areas, but to failure of the treatment to eradicate the fungus from the plots. This might be explained in several ways: (1) It might be assumed that the fungus survived as sclerotia or on roots located at a greater depth than that reached effectively by fungicides applied

in holes 6 inches deep; or (2) it might be that even at the depths reached by the fungicides they were not able to kill the fungus under all the soil conditions encountered; or (3) continuing evaporation of the fungicides from the surface soil might have prevented an effective concentration from being maintained, particularly at points fairly near the surface. Results in line with this third hypothesis were obtained in laboratory experiments with open jars exposed to a current of air (*1, experiment C2O*). Similarly, Godfrey and his associates (*3, 4*) found in experiments for the control of nematodes, that to prevent rapid evaporation of chloropicrin from the soil it was necessary to confine it by a cover of some impervious material. Paper coated with glue was more satisfactory than other soil coverings tested.

It seems possible, then, that failure of tetrachlorethane and xylol to eradicate root rot in the treated plots may have been due more to evaporation of the fungicides from the surface than to failure of these materials to be sufficiently fungicidal or to penetrate the soil adequately. Further experiments might show that covering the surface of the soil would so increase the efficiency of these materials that they would be able to eradicate the root rot. Meanwhile, despite the promising results obtained from applications of these fungicides around infected plants, the other results recorded do not justify recommending them for practical use against *Phymatotrichum* root rot.

SUMMARY

In field tests against root rot caused by *Phymatotrichum omnivorum*, pentachlorethane, tetrachlorethane, and xylol were applied in holes pierced to a depth of 6 inches in the soil, and covered only by pushing soil into the holes.

These materials were applied first in small plots around infected cotton plants and chinaberry and elm trees. After various periods, the efficacy of the materials in killing the fungus on roots was determined by excavating the roots to a depth of 2 feet or more and culturing portions in jars of soil. Roots recovered from plots treated at 166 g per square foot yielded *Phymatotrichum* growth from only 1 of 253 portions; plots treated at 83 g per square foot from 2 of 210 portions; plots treated at 41.5 g per square foot from 7 of 79 portions; and untreated check plots from 92 of 293 portions. The fungicides were effective to at least 2 feet below the depth at which they were applied.

Xylol and tetrachlorethane were applied at 249 g per square foot in an experiment designed to test the ability of these materials to eradicate root rot from infested plots. The plots were bordered by "sorghum barriers" to prevent re-encroachment of root rot from adjoining untreated areas. Incidence of root rot was markedly delayed and its prevalence reduced during the season following treatment, but the disease recurred in each plot from isolated centers of infection.

Applications around growing trees and shrubs showed that tetrachlorethane was rapidly and severely injurious to host plants, xylol next, and that pentachlorethane was least injurious. In general, applications at 166 or 83 g per square foot injured some of the plants, but no injury was found after treatment at 41.5 g per square foot.

The order of susceptibility to fungicide injury, of the plants tested, was approximately as follows, the more susceptible being listed first: Live oak, elm, ilex, hawthorn, hackberry, pomegranate, and retama. Residual injurious effects on crops planted later were much more severe after treatment of soil with tetrachlorethane than after treatment with xylol.

Failure of tetrachlorethane and xylol to eradicate root rot completely from infested plots was possibly due more to loss of the fungicides from the surface soil than to failure of these materials to be sufficiently fungicidal or to penetrate the soil adequately. Further experiments with the surface of the soil covered to lower evaporation losses are suggested. Meanwhile, the results do not justify recommendation of these soil fungicides for practical use against *Phymatotrichum* root rot.

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A CYTOLOGICAL STUDY OF HOST-PARASITE RELATIONS OF VENTURIA INAEQUALIS ON APPLE LEAVES[1]

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INTRODUCTION

Venturia inaequalis (Cke.) Wint. is representative of a group of phytopathogenic fungi characterized by a very distinctive type of parasitism. Though its invading mycelium is characteristically limited to a subcuticular position, nevertheless the fungus has intimate relations with the underlying host tissues, from which it derives substantial nutriment and upon which it exerts important influences. The end results of infection in terms of the efficiency of fungous nutrition and the range of congeniality of host-parasite relations are very similar to those of invasion by many of the so-called obligate parasites.

Though excellent cytological studies have been made on the host-parasite relations of various phytopathogenic fungi, especially the Uredinales (e. g., *3*, *4*, *24*, *27*, *28*, *29*),[2] comparatively little attention has hitherto been given to similar studies within the group of which *Venturia inaequalis* is typical. The classical contributions of Aderhold (*1*, *2*) laid the foundation for such investigations. Wiltshire's (*33*) excellent study substantially supplemented Aderhold's work, especially with reference to the details of penetration of the cuticle and the early phases of establishment of the parasite in the subcuticular position. Little detailed consideration, however, has been given to the distinctive phenomena of parasitism and pathogenesis in the later stages of the host-parasite relations.

The investigation reported herein (*15*) was undertaken in the hope of contributing to the description and interpretation of the phenomena of parasitism of *Venturia inaequalis* and the resistance offered by certain of its hosts. This work is an outgrowth of a series of studies of apple scab and related problems that has been in progress at Wisconsin for some years (*7*, *8*, *11*, *12*, *14*, *16*, *17*, *19*, *20*, *21*, *32*), and is a companion study to an investigation on apple rust (*18*), in which is given a brief review of literature pertinent to both lines of work. Discussions of literature relating especially to the initial stages of infection by *V. inaequalis* are given by Wiltshire (*33*) and Keitt and Jones (*14*). Brown and Harvey (*6*), Brown (*5*), and Rice (*23*) have reviewed the literature of the broader field of host-parasite relations.

MATERIALS AND METHODS

Two monoconidial isolates of *Venturia inaequalis* were employed. These cultures were originally isolated by Palmiter (*20*), and his designations are retained in this paper. Isolate 17 was obtained from

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[2] Reference is made by number (italic) to Literature Cited, p. 617.

infected Fameuse apples (*Malus sylvestris* Mill.) and 22 from infected Delicious apples, both from Wisconsin orchards. These isolates were chosen because they sporulated well on artificial culture media and differed in their capabilities of infecting the foliage of certain apple varieties.

Having been carried in culture for several years without host passage, each isolate was inoculated on apple leaves, and monoconidial reisolations were made by Keitt's (*10*) method. The reisolates conformed closely to Palmiter's descriptions, except that they sporulated more abundantly in culture and seemed to show certain differences from his results in degree of infection. The latter are attributable in part to differences in the methods of classification employed. However, the possibility that the isolates may have undergone some change in culture is recognized.

The apple varieties employed were Fameuse, Yellow Transparent, and Missouri Pippin. These had been included in Palmiter's infection studies and found to react differently when inoculated with the two isolates mentioned above. Preliminary infection experiments with the reisolates showed that these varieties were suitable for the present investigation.

Two-year-old nursery apple trees were placed in galvanized-iron containers in about 8 kg of soil (3 parts compost soil and 1 part sand), adjusted, and held at 70 percent of the maximal water-holding capacity (*31*, v. 1, p. 150). After being rooted in a cool basement for 2 to 3 weeks, they were placed on a greenhouse bench. All trees were kept in the same greenhouse under comparable environmental conditions. Two shoots were allowed to develop on each tree. When the shoots were about 15 to 20 inches long and showed 14 to 18 leaves, they were inoculated. At the time of inoculation, a bit of string was tied about the petiole of the youngest leaf of each shoot to facilitate identification of the inoculated leaves after further growth had occurred. Fumigation was employed as necessary to control insects.

The spores used for inoculation were produced by cultures grown on cheesecloth "wicks" saturated with nutrient solution in 12-ounce medicine bottles. A 15- by 8-cm strip of cheesecloth was placed in each bottle with about 10 cc of nutrient solution (3 percent Trommer's malt extract), so that it formed a closely adherent lining for one of the walls. The bottles were then sterilized in an autoclave. About 10 days before the spores were needed, the sterile nutrient solution in the bottles was seeded with a few drops of a suspension of conidia, introduced by means of a sterile pipette. The bottles were laid in a horizontal position for about 30 minutes to allow the spores to settle on the cheesecloth, then placed upright in an incubator at 20° C. for 6 days, and after that transferred to a 16° incubator where they remained for from 4 to 6 days. The lower temperature favored sporulation.

When a spore suspension was needed for inoculation, the pieces of cheesecloth were removed from the bottles and the spores washed off by atomizing with water. The suspension thus obtained was centrifuged, the supernatant liquid decanted, and water added. In this way a suspension of washed spores relatively free from fungal staling products or nutrient materials could be obtained in any desired concentration. Sterile distilled water and sterile glassware and atomizers were used.

During the greenhouse season six series of inoculations were made, the first on March 23 and the last on May 6. Series 1, 2, 3, 4, and 6 were made with conidia from culture. Series 5 was made with spores washed from sporulating scab lesions on apple leaves infected with the designated isolates for comparison. In each case the inoculations with the different isolates were made separately and suitable precautions were taken to insure against contamination.

Each inoculation was performed in duplicate or quadruplicate, depending on the material available. With the exception of series 2 and 4, four trees of each variety were inoculated with each isolate. In each lot 2 trees were heavily inoculated (a drop of spore suspension when placed on a glass slide under the microscope showing approximately 150 spores per low-power field) and the remaining 2 were lightly inoculated (10 spores per low-power field). The heavily inoculated leaves were intended for study of the early stages of infection, whereas the light inoculation was planned for studying the older lesions. One of the lightly inoculated trees of each isolate-variety combination was reserved for study of macroscopic development of the disease.

Prior to the application of inoculum each tree was thoroughly washed with a fine spray of tap water from an angled spray nozzle, gently shaken to remove the larger drops, and allowed to dry.

The spore suspensions were applied to the upper, or ventral, surface of all the leaves on each shoot by means of a De Vilbiss atomizer which had previously been throughly cleaned and steamed for 15 minutes. Only one tree was inoculated at a time in a room remote from the greenhouse in which the remaining trees were kept. After inoculation the trees were placed for 36 hours in a moist chamber described by Keitt et al. (*13*, fig. 20) at 16° C. They were then returned to the greenhouse.

Fixations were made at regular intervals after inoculation. Material was collected daily for the first 6 days and at longer intervals for the next 4 weeks. Two earlier collections were made at 14- and 20-hour intervals, respectively, after inoculation in order to obtain the early stages of penetration. To promote uniformity of results daily fixations were made at 4 p. m. and material was always taken from the third or fourth leaf below the one marked with string.

In a preliminary experiment, various fixatives were tried in combinations with different methods of dehydrating and staining. A formal-chrom-acetic mixture (1-percent aqueous solution of chromic acid, 100 cc; glacial acetic acid, 4 cc; 40-percent aqueous solution of formaldehyde, 50 cc; freshly prepared for each fixation) suggested in correspondence by G. H. Conant gave the best results and was used throughout. The n-butyl alcohol method of Zirkle (*34*) was employed for dehydration and embedding. Sections were cut 6μ thick. Heidenhain's iron-alum haematoxylin was a satisfactory stain for the early stages of penetration because it brought out the infection hypha clearly. A modification of Flemming's triple stain consisting of safranine, gentian violet, and fast green was best for the study of host-parasite reactions. In studying the relationships of the fungus to the cuticle and the walls of the epidermal cells, sections stained in Delafield's haematoxylin and Sudan IV and mounted in glycerin were useful.

A modification of the cleared-leaf method of Peace (*22*) was well adapted to a survey of certain aspects of conidial germination, direct cuticular penetration, and the establishment of the fungus beneath the cuticle, as it afforded a means of observing these phenomena in toto on relatively large areas of leaf surface. Bits of leaf were collected 14, 24, 48, 96, and 144 hours, respectively, after inoculation, fixed in acetic alcohol, cleared in chloral hydrate, and stained with acid fuchsin in lactophenol.

After each inoculation, material was collected for fixation and clearing as described above. The data reported in this paper, however, are taken chiefly from series 3 because of the very favorable circumstances that attended this experiment. The trees were growing uniformly and vigorously and were entirely free from greenhouse insects.

MACROSCOPIC OBSERVATIONS ON LIVING LEAVES

At suitable intervals after inoculation, data were taken on the appearance and development of infection on the individual leaves of one shoot from each of the trees that were set aside for this purpose. The results, shown in table 1, are also representative of the infection that developed on the trees from which material was taken for microscopic study.

Isolate 22 was highly pathogenic on Yellow Transparent, strikingly less virulent on Fameuse, and only slightly pathogenic on Missouri Pippin.

On the young leaves of Yellow Transparent, infection became macroscopic the ninth day following inoculation, and the incubation period on the older leaves was progressively longer (*14*). These spots, at first small, indistinct, and slightly olivaceous, increased rapidly in diameter and began to produce conidia abundantly on about the fourteenth day after inoculation. Necrosis began to appear in the middle of these lesions about 5 days after the first sporulation. The necrotic area spread rapidly for the next 10 days, after which nearly all of the visible lesion was dead. Necrosis always followed sporulation and neither phenomenon occurred on leaves below the sixth (the one marked with string being no. 1). Development apparently ceased in all the lesions in from 30 to 36 days after inoculation.

On Fameuse, infection first appeared about 12 days after inoculation, and was confined to the younger leaves near the tip of the shoot. The lesions remained small (less than 5 mm in diameter), produced no conidia, and necrosis was not apparent. About 3 weeks after inoculation, development apparently ceased, and the lesions began to change somewhat, becoming bronzed or reddish brown.

On the leaves of Missouri Pippin, no macroscopic signs of infection were detected until 18 days after inoculation, when minute, barely visible flecks appeared. These flecks seemed to be more or less grouped together in small patches a millimeter or less in diameter. These manifestations might easily have been confused with certain insect punctures. Because the plants studied were free from insects, however, it was thought, and later proved by the cytological study, that the flecks actually were scab lesions.

The symptoms that developed when isolate 17 was used with the same three varieties were distinctly different from those just described.

TABLE 1.—*Occurrence of macroscopic lesions on the ventral surface of apple leaves of series 3, inoculated with Venturia inaequalis, Apr. 17, 1935,*

Isolate and variety	Dates noted	Lesions on stated leaves, numbered serially from base to tip of shoot																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number
22, Yellow Transparent	Apr. 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	(1)	(2)	(2)	------
	Apr. 29	0	0	0	0	0	0	0	0	0	0	0	8	26	(2)	(2)	(2)	(2)	------
	May 1	0	0	0	0	0	0	0	0	4	17	14	26	(1)	(2)	(2)	(2)	(2)	------
	May 5	0	0	0	0	0	0	0	7	28	(1)	(1)	(1)	(1)	(2)	(2)	(2)	(2)	------
	May 10	0	0	0	0	0	8	0	7	(1)	(1)	(1)	(1)	(1)	(2)	(2)	(2)	(2)	------
	May 16	0	0	0	0	0	8	13	17	(1)	(1)	(1)	(1)	(1)	(2)	(2)	(2)	(2)	------
	May 25	0	0	0	0	0	8	13	17	(1)	(1)	(1)	(1)	(1)	(2)	(2)	(2)	(2)	------
17, Yellow Transparent	Apr. 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	------	------
	Apr. 29	0	0	0	0	0	0	0	0	0	0	0	0	0	8	(1)	------	------	------
	May 1	0	0	0	0	0	0	0	0	0	0	0	0	0	(1)	(2)	------	------	------
	May 5	0	0	0	0	0	0	0	0	0	0	7	33	4	(1)	(2)	------	------	------
	May 10	0	0	0	0	0	0	0	0	0	(1)	16	40	(1)	(1)	(2)	------	------	------
	May 16	0	0	0	0	0	0	0	0	0	(1)	(1)	(1)	(1)	(1)	(2)	------	------	------
	May 25	0	0	0	0	0	0	0	0	0	(1)	(1)	(1)	(1)	(1)	(2)	------	------	------
17, Fameuse	Apr. 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Apr. 29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)
	May 1	0	0	0	0	0	0	0	0	0	0	0	0	26	(1)	(1)	(2)	(2)	(2)
	May 5	0	0	0	0	0	0	0	0	0	0	15	16	(2)	(2)	(2)	(2)	(2)	(2)
	May 10	0	0	0	0	0	0	0	0	12	33	(1)	(1)	(2)	(2)	(2)	(2)	(2)	(2)
	May 16	0	0	0	0	0	0	0	0	(1)	(1)	(1)	(1)	(2)	(2)	(2)	(2)	(2)	(2)
	May 25	0	0	0	0	0	0	0	0	(1)	(1)	(1)	(1)	(2)	(2)	(2)	(2)	(2)	(2)
22, Fameuse	Apr. 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	------	------	------
	Apr. 29	0	0	0	0	0	0	0	0	0	0	0	26	(1)	(1)	------	------	------	------
	May 1	0	0	0	0	0	0	0	0	0	0	0	(1)	(2)	(2)	------	------	------	------
	May 5	0	0	0	0	0	0	0	0	0	0	10	(1)	(2)	(2)	------	------	------	------
	May 10	0	0	0	0	0	0	0	0	0	0	24	(1)	(2)	(2)	------	------	------	------
	May 16	0	0	0	0	0	0	0	0	0	0	24	(1)	(2)	(2)	------	------	------	------
	May 25	0	0	0	0	0	0	0	0	0	0	24	(1)	(2)	(2)	------	------	------	------

See footnotes at end of table.

TABLE 1.—*Occurrence of macroscopic lesions on the ventral surface of apple leaves of series 3, inoculated with Venturia inaequalis, Apr. 17, 1935*—Continued

Isolate and variety	Dates noted	Lesions on stated leaves, numbered serially from base to tip of shoot																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
		Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number	Number
17, Missouri Pippin	Apr. 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	------
	Apr. 29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	------
	May 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	(1)	(1)	------	------
	May 5	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)	(2)	(2)	------	------
	May 10	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)	(2)	(2)	------	------
	May 16	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)	(2)	(2)	------	------
	May 25	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)	(2)	(2)	------	------
22, Missouri Pippin	Apr. 26	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	------	------
	Apr. 29	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	------	------
	May 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	------	------	------
	May 5	0	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)	------	------	------
	May 10	0	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)	------	------	------
	May 16	0	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)	------	------	------
	May 25	0	0	0	0	0	0	0	0	0	0	0	0	0	(2)	(2)	------	------	------

[1] More than 50 lesions.
[2] Lesions numerous, but not clearly enough defined to count.

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TABLE 2.—*Data pertaining to the inception of infection of apple leaves by Venturia inaequalis in the several isolate-variety combinations studied* [1]

Isolate and variety	Development after—																
	14 hours			24 hours					48 hours					96 hours				
	Germinated	Appressoria	Pores	Germinated	Apressoria	Pores	Primary stromata	Average diameter of primary stromata	Primary stromata	Average diameter of primary stromata	Secondary hyphae	Average diameter of lesions	Epidermal cells necrotic	Primary stromata	Average diameter of primary stromata	Lesions	Average diameter of lesions	Epidermal cells necrotic
	Number	*Number*	*Number*	*Number*	*Number*	*Number*	*Number*	*Microns*	*Number*	*Microns*	*Number*	*Microns*	*Number*	*Number*	*Microns*	*Number*	*Microns*	*Number*
22, Yellow Transparent	38	0	0	47	55	51	22	18.5	66	55	362	176	0	59	75.3	46	356.6	0
17, Yellow Transparent	40	0	0	46	66	62	4	9.0	75	25.7	236	90.5	0	71	37.7	45	223.0	0
17, Fameuse	44	0	0	50	80	73	41	13.0	68	41.0	297	137.3	0	76	55.4	49	341.0	0
22, Fameuse	34	0	0	46	51	49	10	7.5	58	17.0	228	114.8	0	65	44.6	46	208.0	0
17, Missouri Pippin	45	0	0	49	77	60	32	10.0	54	23.3	178	92.3	0	59	36.4	46	193.0	0
22, Missouri Pippin	37	0	0	43	48	46	40	6.0	51	19.0	129	90.8	86	58	37.3	42	164	116

[1] In each case the data were from 50 conidia taken at random on the ventral surface of young cleared leaves studied in toto.

In the case of Yellow Transparent and Fameuse, the degree of severity of infection by isolate 17 was just the converse of that by 22, 17 being intermediately or mildly parasitic on the leaves of Yellow Transparent and highly parasitic on Fameuse. Infections by 17 on Fameuse and 22 on Yellow Transparent were closely similar with reference to incubation period, sporulation, and general development and appearance of the lesions. Infections by 17 on Yellow Transparent and 22 on Fameuse were also comparable in the degree of development. Missouri Pippin was decidedly resistant to isolate 17 as well as 22, although the manifestations of resistance were different in these two isolate-variety combinations. As mentioned above, isolate 22 induced minute flecks on Missouri Pippin leaves about 18 days after inoculation. In contrast, isolate 17 on the leaves of this variety induced macroscopic lesions in from 12 to 15 days after inoculation. These lesions were irregular and diffuse in outline and attained a diameter of 1 cm or more. They were pale green and easily discernible, in contrast to the dark-green color of the normal Missouri Pippin leaf. Apparently there was no sporulation or development of necrosis.

EARLY STAGES OF INFECTION

CLEARED-LEAF STUDY

The phenomena of the germination of conidia, formation of appressoria, and penetration of the cuticle, as observed on cleared leaves examined in toto, appeared to be similar in all the isolate-variety combinations. Following penetration, however, there were distinctive differences in the extent and nature of the development of the mycelium in the subcuticular position. Results from microscopic counts and measurements made on material of each isolate-variety combination collected at intervals of 14, 24, 48, and 96 hours, respectively, after inoculation are shown in table 2.

At the end of the 14-hour period, germination of most of the conidia had begun. The conidia of both isolates were usually two-celled. In this early stage of germination, the distal cell of the spore had expanded and developed a slight protuberance. The spore wall, however, remained intact, forming a thin, slightly stained membrane about the protuberance.

At the end of the 24-hour period, the process was decidedly farther advanced. The rudimentary germ tubes, usually from the distal cell as described above, had bulged at the tip to form characteristic appressoria, closely adherent to the cuticle. Occasionally a germ tube branched to form two distinct appressoria, each of which functioned in actual penetration of the host. Penetration from the proximal cell of the conidium was less common. It was observed to take place in some cases without the development of a germ tube or characteristically differentiated appressiorium (*14*), the cell itself being modified to function as an appressorium (fig. 1, *D*).

EXPLANATORY LEGEND FOR FIGURE 1

FIGURE 1.—Germination of conidia, formation of appressoria, and early development of infection from cleared leaves examined in toto: *A*, Isolate 22 on Yellow Transparent 24 hours after inoculation; spore (*s*), appressorium with penetration pore (*a*), and young amoeboid primary hypha (*ph*). × 900. *B*, Isolate 22 on Yellow Transparent 36 hours after inoculation; proliferation of cells in primary hypha (*ph*), young stolonlike hyphae (*st*). × 900. *C*, Isolate 22 on Yellow Transparent 4 days after inoculation; primary fungal stroma (*ps*), secondary lateral branches forming at *sb*, stolonlike hyphae (*st*), lateral branch initials (*b*), and secondary stromata (*ss*). × 400. *D*, Isolate 17 on Missouri Pippin 4 days after inoculation; primary stromata (*ps*) and stolonlike branches (*st*) less vigorous in appearance than those in *C*. × 400. *E*, Isolate 22 on Missouri Pippin 2 days after inoculation; epidermal cells showing necrosis are represented in solid black. × 400. *F*, Isolate 22 on Missouri Pippin 4 days after inoculation; epidermal cells showing necrosis are represented in solid black. × 400.

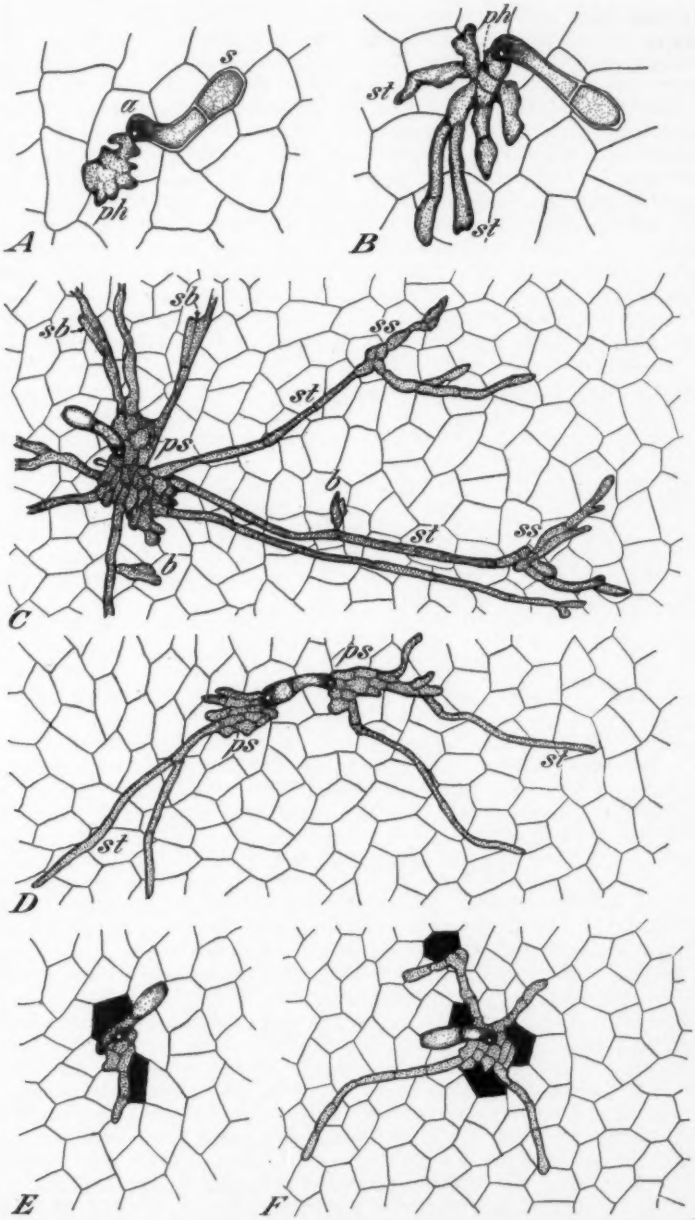


FIGURE 1.—Germination of conidia, formation of appressoria, and early development of infection from cleared leaves examined in toto. (For explanatory legend see opposite page.)

In the wall of the appressorium in contact with the leaf surface a characteristic transparent porelike area, usually about 2μ in diameter (p. 603), indicated the place of direct cuticular penetration. In some preparations very careful focusing with an oil-immersion objective seemed to show the presence of the infection hypha itself at or near the middle of the transparent area. This image, however, was not distinct enough to be interpreted with absolute certainty. The appearance of the primary hypha beneath the cuticle, however, was definite proof that penetration had occurred. At the end of the 24-hour period, the isolate-variety combinations of 22 on Yellow Transparent and Missouri Pippin and 17 on Fameuse and Missouri Pippin showed high percentages of completed penetration. In cleared-leaf preparations, the primary hypha was hyaline and only very slightly stained with the acid fuchsin. Unless it protruded beyond the overlying appressorium, it was masked by this structure and, consequently, overlooked. In view of this fact, the figures in table 1 recording the occurrence of primary hyphae represent the minimal rather than the maximal number of actual entries that had occurred at the time. A typical case showing a germinating conidium, appressorium, pore and primary hypha is shown in figure 1, *A*.

The single-celled, irregularly shaped primary hyphae, which were found in most 24-hour-old preparations, rapidly increased in size, due to cell proliferation and enlargement, and developed into primary fungal pads or stromata. Secondary hyphal branches radiated out from the periphery of the primary stromata. A 36-hour preparation (fig. 1, *B*) illustrates these phenomena.

Data taken at the end of the 48-hour period show that the development of each isolate on its most susceptible host variety had markedly exceeded that of the same isolate on less susceptible varieties. For example, with reference to the size of the primary stromata, the number of secondary hyphae, and the diameters of the lesions, isolate 22 on Yellow Transparent, closely followed by 17 on Fameuse, was much more advanced than 17 on Yellow Transparent or 22 on Fameuse. Thus far, each of the isolates had developed only very feebly on the resistant variety Missouri Pippin. With isolate 22 on this variety, epidermal cells underlying the spreading hyphae showed brown discoloration, a manifestation of necrosis that will be discussed in detail later. The position of the collapsed epidermal cells in relation to the feeble, somewhat gnarled hyphae is shown in the two camera lucida sketches in figure 1, *E* and *F*. Isolate 17, however, did not induce collapse of the underlying epidermal cells (fig. 1, *D*).

In contrast to their feeble mycelial growth on Missouri Pippin, both isolates developed in a typically dendritic fashion on Yellow Transparent (fig. 1, *C*) and Fameuse. Proliferation of the cells of the primary stroma (*ps*) continued and from its periphery long slender, stolonlike hyphae (*st*) radiated outward. These hyphae at first were single and unbranched, with widely separated septa. Lateral branches arose at very sharp angles as shown at *sb* or at right angles (*b*). The former type radiated out in the same fashion as the stolonlike secondary hyphae, while the latter type developed feebly to intensify the network or lattice arrangement of the fungal pattern. After the stolonlike hyphae had reached a distance of from 50μ to 100μ from the primary stroma, secondary stromata were produced. From these, tertiary branches radiated still farther. This process was repeated

several times during the spread of the lesion. In the isolate-variety combinations of 22 on Yellow Transparent and 17 on Fameuse, proliferation of the cells of these individual stromata often resulted in the development of a structure several cells thick, and it was from these stromata that conidiophores penetrated the cuticle and produced spores in large numbers. In the combinations of 22 on Fameuse and 17 on Yellow Transparent, many conidiophores were formed but no conidia were observed.

At the end of the 96-hour period the most significant differences were apparent in the average diameter of the lesions. The differences that have been pointed out with reference to the material collected at the end of the 48-hour period were increased. In addition, the hyphae were robust in all lesions except on Missouri Pippin, on which they were slender and feeble.

PHENOMENA OF PENETRATION

The germinating conidia of *Venturia inaequalis* penetrated the cuticle of the apple leaf directly. The process was similar in all details for all isolate-variety combinations. There was no cytological evidence to show that the cuticular barrier of young leaves in any way hindered the establishment of the fungus, even in the least compatible isolate-variety combinations studied.

Actual cuticular penetration as well as the sequence of events preceding it were best observed and interpreted in preparations of material collected 20 hours after inoculation. For convenience several processes involved in penetration will be described separately as follows:

Germination of Condium

As described under the "cleared-leaf study" the germ tubes were very short or lacking and were usually observed as mere protuberances from the distal, or pointed, end of the spores. During the germination process, each cell of the spore contained one nucleus (fig. 2, *G*). The distal, or germinating, cell became densely filled with protoplasm, whereas the proximal cell appeared to be partly evacuated (fig. 2, *G, I, K, L*).

Formation of Appressorium

The advancing tip of the germ tube soon became closely adherent to the leaf surface and developed into a more or less clearly differentiated appressorium. Viewed in cross section, the appressoria appeared to be oval or rounded in outline and densely filled with cytoplasm (fig. 2, *A, B*). The protoplasm stained heavily, and in some cases the nuclei could not be identified. Longitudinal sections (fig. 2, *C, E, F, G, K*) clearly show that the appressorium is a swollen, modified germ tube tip, closely appressed to the cuticle. In many cases, owing to the retardation of apical elongation because of the tenacious adherence of the appressorium, the germ tube arches as shown in figure 2, *C, E, F, G, H, J*.

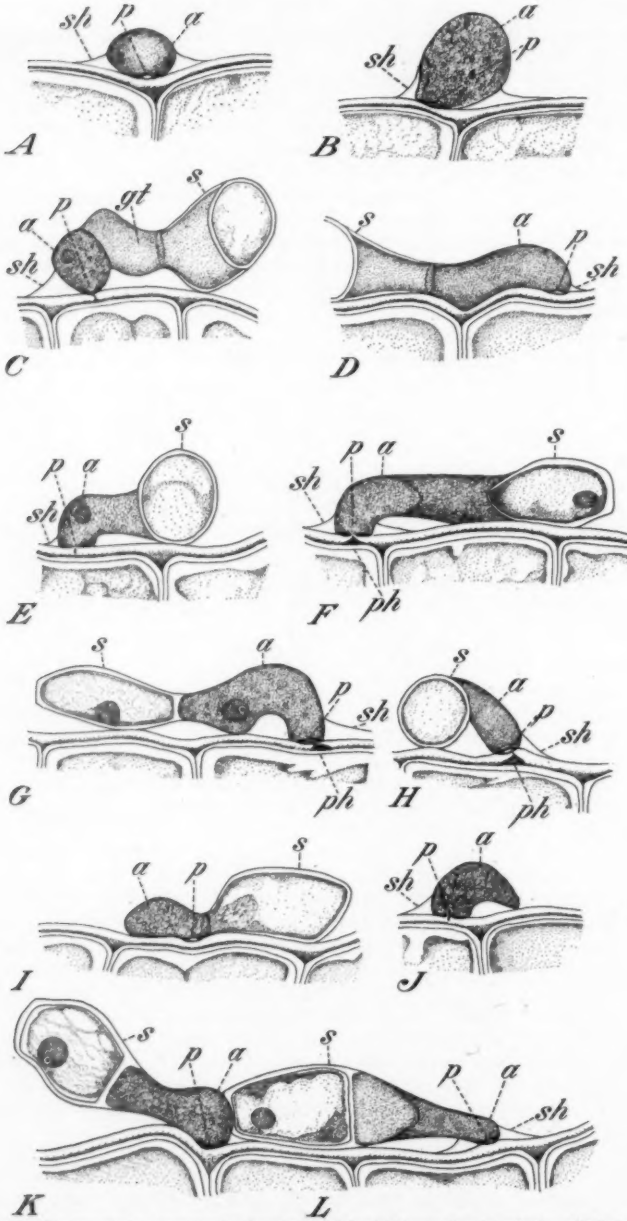


FIGURE 2.—Various stages of penetration from material fixed 20 hours after inoculation with isolates 22 and 17: *A*, 22 on Missouri Pippin; *B*, 22 on Fameuse; *C*, 22 on Yellow Transparent; *D*, 17 on Missouri Pippin; *E–G*, 22 on Fameuse; *H*, 22 on Yellow Transparent; *I*, 22 on Missouri Pippin; *J*, 22 on Fameuse; *K–L*, 17 on Missouri Pippin. Spore, *s*; germ tube, *gt*; appressorium, *a*; mucilaginous sheath, *sh*; pore, *p*; primary hypha, *ph*. × 1,620.

Mucilaginous Sheath

The appressoria were apparently held fast to the leaf surface by a mucilaginous sheath. The fixed remains of this structure were usually found in the cytological preparations (fig. 2, *A*, *B*). The sheaths, however, could not be demonstrated in connection with germinating conidia on glass slides by the dilute india-ink method, and they were not seen with certainty in the cleared-leaf preparations (*14*, *33*).

Circular Thickening and Pore

A circular, disklike area of thickening, usually about 6μ in diameter, occurred in the wall of the appressorium that was in contact with the leaf surface. In the middle of this structure was a circular, transparent, thin-walled, porelike area about 2μ in diameter. For convenience, these structures are subsequently referred to as the circular thickening and the pore (fig. 1, *A*, *B*, and fig. 2, especially *A*, *B*, *D*, *H*, *I*).

Infection Hypha

At or near the center of the pore, a minute infection hypha pierced directly downward or slightly obliquely through the cuticle (fig. 2, *C*, *E*, *G*, *J*). Such structures appeared in most of the 20-hour-old preparations in each isolate-variety combination. None was found in 14-hour preparations nor in 48-hour or older ones. In view of these facts and taking into account the stage of development in most 20-hour preparations, it is assumed that actual penetration began about 16 to 18 hours after inoculation, and that the infection hyphae disappeared or were not sufficiently stained to be seen shortly after infection had been established.

Primary Hypha

As soon as the infection hypha reached the epidermal cell wall it began to flatten into an irregularly shaped primary hypha (fig. 2, *C*, *F*, *G*, *H*). Further development of the fungus and the reactions of the underlying host tissues were different in the various isolate-variety combinations. Consequently, they will be discussed separately.

DEVELOPMENT OF INFECTION

A description of phenomena resulting from the different host-parasite reactions in the several isolate-variety combinations follows. The two combinations that favored aggressive parasitism are discussed first, and the reciprocal combinations, in which there was a strikingly lower degree of parasitism, are treated comparatively. The combination of each isolate with the resistant variety is discussed separately, because the host-parasite relations were decidedly different in the two cases.

AGGRESSIVE PARASITISM

Isolate 22 was aggressively parasitic on Yellow Transparent and 17 was equally aggressive on Fameuse. These infections were characterized by the vigorous development of the fungal hyphae, the formation of relatively thick stromata in the subcuticular position, prolific

sporulation of the parasite, and extreme impoverishment of the underlying host tissues, culminating in their death and collapse.

As previously described in the cleared-leaf study, the primary hyphae were at first single-celled and somewhat amoeboid or irregular in shape. A 24-hour-old preparation (fig. 3, *E*) shows that the primary hypha (*ph*) has expanded until it nearly covers the underlying epidermal cell and appears to contain a nuclear division figure, probably its first. A prominent nucleus remains in the appressorium (*a*). The underlying host tissues show no ill effects of fungal invasion. Another 24-hour-old infection (fig. 3, *B*) shows a young stroma developing beneath the thick cuticular layer in an epidermal hair socket. Many instances of penetration at the base of epidermal hairs were encountered.

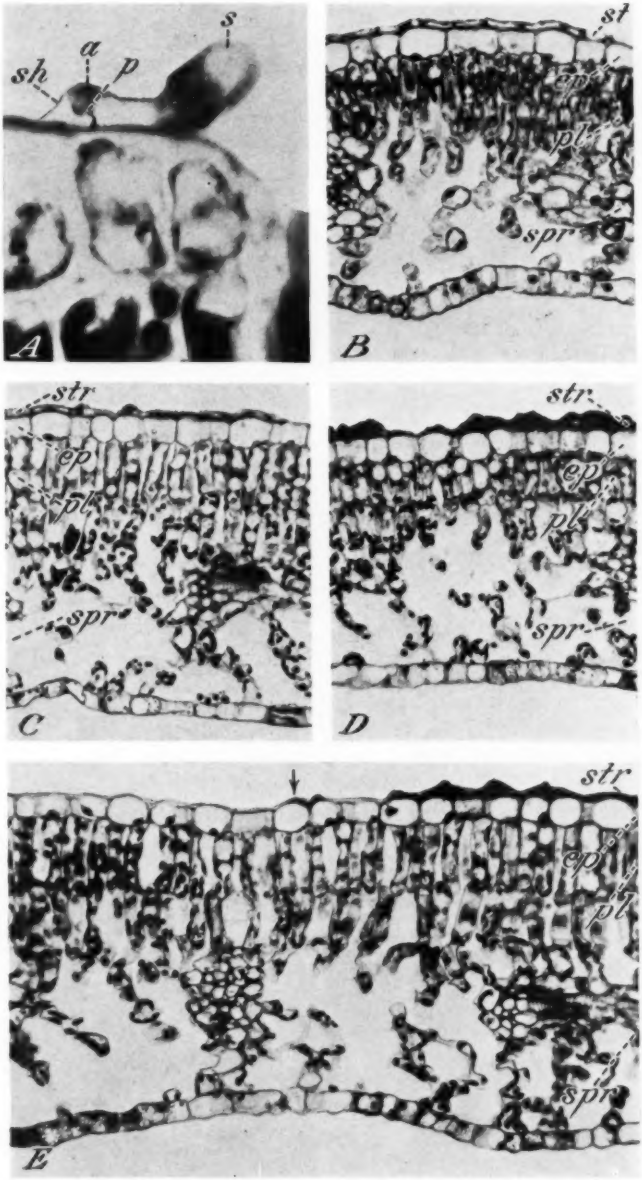
A median section of a 2-day-old lesion (fig. 3, *A*) illustrates the rapid proliferation of the primary hypha to form a multicellular stroma (*ps*), from which a slender stolonlike branch (*st*) is beginning to radiate. The fungal cells are uninucleate, the nuclei being comparatively large and prominent. These young fungal cells in the primary stroma, although appearing hyaline in cleared-leaf preparations, are filled with very dense cytoplasm which stained heavily in cytological preparations. In fact, in some preparations the cytoplasm is so dense that it masks the nuclei almost completely. The host nuclei in the underlying epidermal cells retain their normal shape and staining reaction. The cells, however, appear to be slightly plasmolyzed. This plasmolysis is attributed to the fixative, rather than to any deleterious fungal activity, because epidermal cells considerably removed from infected regions are similar in this respect.

Throughout the incubation period, during which the fungus became firmly established and widespread in the subcuticular region, there was marked congeniality between the parasite and host. The fungus developed very rapidly and the underlying epidermal and palisade cells remained normal in appearance. The host tissues beneath a vigorously expanding stolonlike hypha in a 6-day-old infection (fig. 3, *F*) are just as normal in appearance as those beyond the area of infection. All nuclei retain their normal structure, staining reaction, and position. In addition, the palisade cells apparently contain the full complement of normal chloroplasts.

Coincident with the macroscopic appearance of the lesions, about 9 or 10 days after inoculation, impoverishment of the upper palisade layer appeared in the middle of the lesion. This depletion was manifested chiefly by the disappearance of plastids and by marked vacuolation as shown in plate 1, *D*.

EXPLANATORY LEGEND FOR PLATE 1

A, Isolate 22 on Yellow Transparent 20 hours after inoculation. The original photomicrograph, taken from the same lot of material as figure 2, *C*, was enlarged approximately × 2 with a photographic projector. Spore (*s*), appressorium (*a*), mucilaginous sheath (*sh*), infection hypha penetrating the unstained cuticle at the pore (*p*). × 800. *B*, Isolate 22 on Yellow Transparent 6 days after inoculation. Stolonlike hypha (*st*) near the periphery of lesion; underlying epidermis (*ep*), palisade (*pl*), and spongy parenchyma (*spr*) apparently normal. × 330. *C*, Isolate 17 on Fameuse 16 days after inoculation; young stroma (*str*) about midway between the center and the periphery of the lesion; upper epidermis (*ep*) and palisade (*pl*) impoverished; spongy parenchyma (*spr*) showing full complement of normal plastids. × 330. *D*, Isolate 17 on Fameuse 10 days after inoculation: Stroma (*str*) in the center of the lesion; upper epidermis (*ep*) and palisade layer (*pl*) vacuolate; spongy parenchyma region (*spr*) normal. × 330. *E*, Isolate 17 on Fameuse 16 days after inoculation: Peripheral stroma (*str*) above impoverished upper epidermis (*ep*) and palisade layer (*pl*); underlying spongy parenchyma region (*spr*) normal; arrow points to extreme peripheral hypha which lies just above the line of demarcation between impoverished and normal host tissues. × 330.



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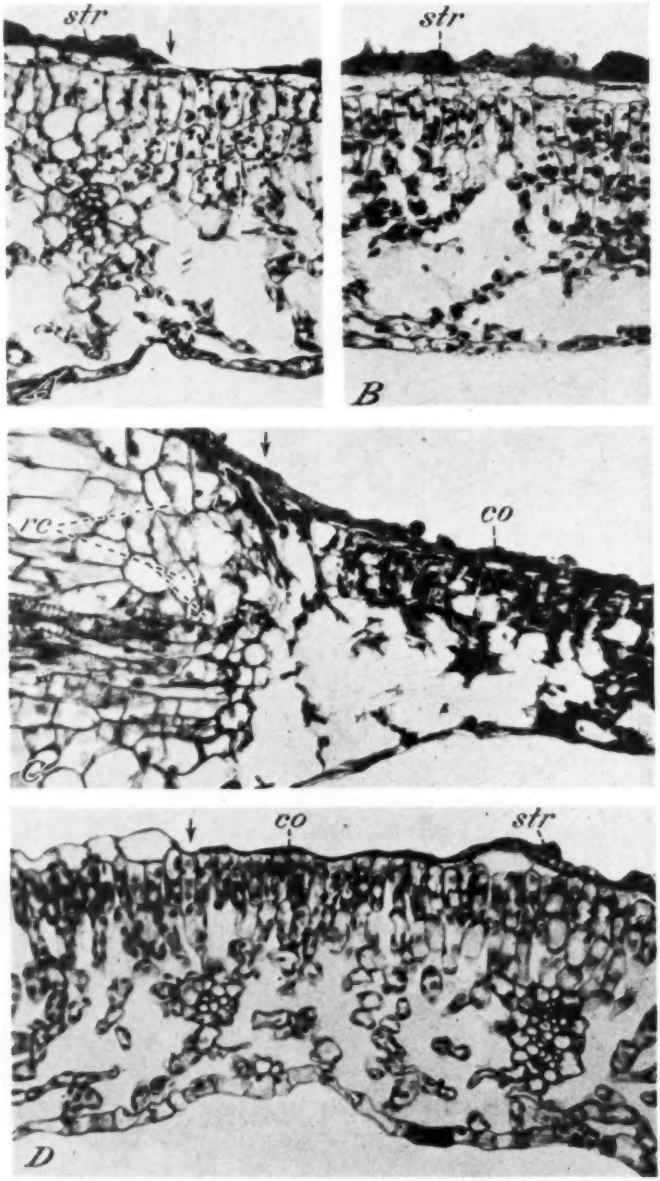

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Impoverishment of the palisade region usually became coextensive with the lesion on the fourteenth or fifteenth day after inoculation. A small portion about midway between the center and the periphery of a 16-day-old lesion is shown in plate 1, *C*. The highly vacuolate upper palisade cells, which are practically devoid of plastids, appear in decided contrast to the spongy parenchyma cells below, which contain a full complement of apparently normal chloroplasts. The margin of a similar lesion (pl. 1, *E*) shows that the region of impoverishment coincides with or even extends a short distance beyond the area of fungal invasion.

Following impoverishment of the host tissues, death and collapse of the affected cells gradually ensued. First, the remaining contents of the cells lost their organized structure and became aggregates of small globules, which appeared to be homogeneous and stained heavily with safranine. These globules coalesced into larger irregularly shaped masses, and finally the cell walls collapsed. The cells later lost their identity completely. Progressive stages of this process are shown in plate 2, *A*, *B*, *C*. In *A*, death of cells is just beginning to occur and is manifested first in the epidermal layer. In the mesophyll tissues beneath, an arrow points to the line of demarcation between the region of extreme impoverishment on the left and that of death and incipient collapse on the right.

In plate 2, *B*, all of the mesophyll cells are dead and their protoplasts disorganized. Very few of the cell walls have collapsed, however, and they still retain their avidity for the acid stain. In *C*, an arrow indicates the margin of a totally collapsed area near the center of a 30-day-old lesion. Rhomboidal cells (*rc*), typical of wound reaction, are formed between the region of collapse and that of extreme depletion.

Injury or death of fungal cells followed the collapse of the host tissues beneath. In the two instances shown in plate 2, *A*, *B*, the subcuticular mycelium (*str*) is still alive while in *C* the mycelium at *co* has collapsed.

When the lesions attained the age of approximately 1 month, spread of infection practically ceased. The typical margin of an old lesion is shown in plate 2, *D*. The region of palisade cell depletion, which is indicated by an arrow, is coextensive with the area invaded by the fungus. The outpost hyphae, however, as well as the epidermal cells (*co*) beneath them, have collapsed. A narrow zone of fungus (*str*) a short distance back from the periphery remains alive. The occurrence of this zone of living fungal tissue is common, but not universal, and is often associated with the larger veins.

EXPLANATORY LEGEND FOR PLATE 2

A, Isolate 17 on Fameuse 21 days after inoculation. Living stroma (*str*) above collapsed upper epidermal layer; arrow points to line of demarcation between region of incipient collapse on the right and that of extreme impoverishment on the left. *B*, Isolate 17 on Fameuse 21 days after inoculation. All mesophyll cells are dead and beginning to collapse; fungal cells of stroma (*str*) still living. *C*, Isolate 17 on Fameuse 30 days after inoculation. An arrow points to the line of demarcation between the region of total collapse on the right and that of extreme impoverishment on the left; rhomboidal cells (*rc*) at the line of demarcation indicate cork formation; collapsed fungal stroma (*co*) associated with necrotic leaf tissue. *D*, Isolate 22 on Yellow Transparent 36 days after inoculation. Arrow indicates margin of lesion, which corresponds with the region of palisade impoverishment; peripheral hyphae and underlying epidermal cells have collapsed at *co*; a narrow zone of fungus (*str*) a short distance back from the periphery remains alive. All × 330.

INTERMEDIATE PARASITISM

In contrast to the manifestations of aggressive parasitism just described, the reciprocal isolate-variety combinations (22 on Fameuse and 17 on Yellow Transparent) showed a significantly lower degree. Fungal development was less vigorous, impoverishment of underlying host tissues was less severe, necrosis of host tissues was much less apparent, and conidia were not produced.

Further study of material from these intermediately parasitic combinations indicated that the host-parasite reactions differed from those of the aggressive combinations in degree rather than in kind. In young infections the cells of the mycelium were robust, normally developed, and apparently unharmed. The underlying host tissues were not noticeably affected until about 14 days after inoculation, when impoverishment of the upper palisade layer began to be evident. In older infections, the impoverished area usually became coextensive with the lesion and involved the whole mesophyll. Necrosis was usually confined to the epidermal layer immediately beneath the fungal stroma. Ultimately the epidermis and superimposed fungal stroma collapsed and lost their identity (pl. 3, *B*).

PHENOMENA OF RESISTANCE

Missouri Pippin was resistant to each of the two isolates, but the host-parasite reactions following infection by 22 were distinctly different from those following infection by 17.

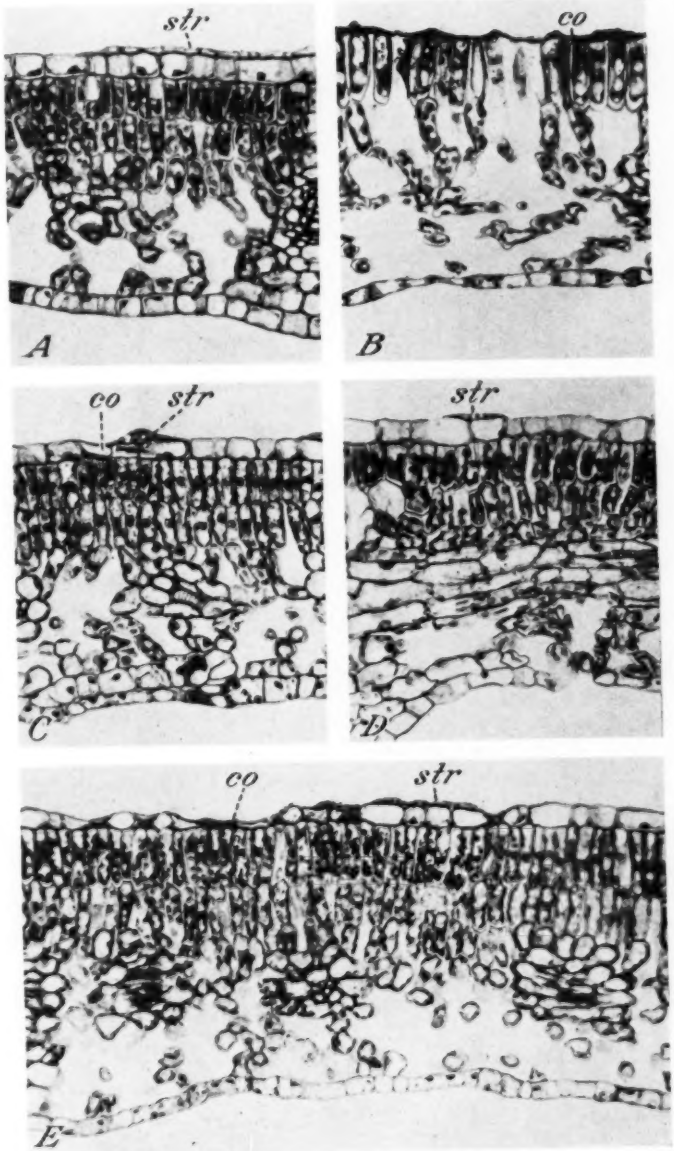
Isolate 22

The epidermal cells of this resistant variety were hypersensitive to attack by isolate 22, collapsing soon after the fungus became established beneath the cuticle. The primary hyphae (fig. 3, *D*, *ph*) of a 2-day-old infection is composed of several small cells, each of which contains one conspicuous nucleus. The appressorium (*a*) is still attached to the cuticle, and the pore (*p*) in its lower wall indicates the point of penetration, although the infection hypha cannot be seen. The epidermal cell (*de*) beneath the primary hypha has collapsed and the nuclei (*in*) in adjacent epidermal cells show signs of disorganization. The palisade cells beneath show no deleterious effects.

The fungus continued to spread feebly, however, sometimes forming a lesion 2 mm in diameter. Along the paths of the rather feebly developed hyphae scattered groups of epidermal cells succumbed. The fact that dead epidermal cells were frequently found near the margin of a lesion is further indication that the cells of the epidermis were highly intolerant of the parasite. A photomicrograph of the central part of a 16-day-old infection (pl. 3, *C*) shows a hyphal strand (*str*) in cross section, and beneath it are two collapsed epidermal cells (*co*). A similar view of a fungal strand in longitudinal section in a 24-day-old infection (pl. 3, *E*) shows that most of the epidermal cells (*co*) along the path of the fungus have died and collapsed.

EXPLANATORY LEGEND FOR PLATE 3

A, Isolate 17 on Missouri Pippin 24 days after inoculation. Longitudinal section of hyphal strand (*str*) near the center of the lesion; underlying mesophyll tissues apparently normal. *B*, Isolate 22 on Fameuse 30 days after inoculation. Fungal stroma and upper epidermis collapsed at *co*. *C*, Isolate 22 on Missouri Pippin 16 days after inoculation. Cross section of fungal strand (*str*) near the center of the lesion; two underlying epidermal cells collapsed at *co*. *D*, Isolate 17 on Missouri Pippin 16 days after inoculation. Cross section of fungal strand (*str*) near the center of the lesion; underlying leaf tissues normal. *E*, Isolate 22 on Missouri Pippin 24 days after inoculation. Longitudinal sections of fungal strand (*str*) near the center of the lesion; fungal cells and epidermis collapsed at *co*; underlying palisade region injured All × 330.



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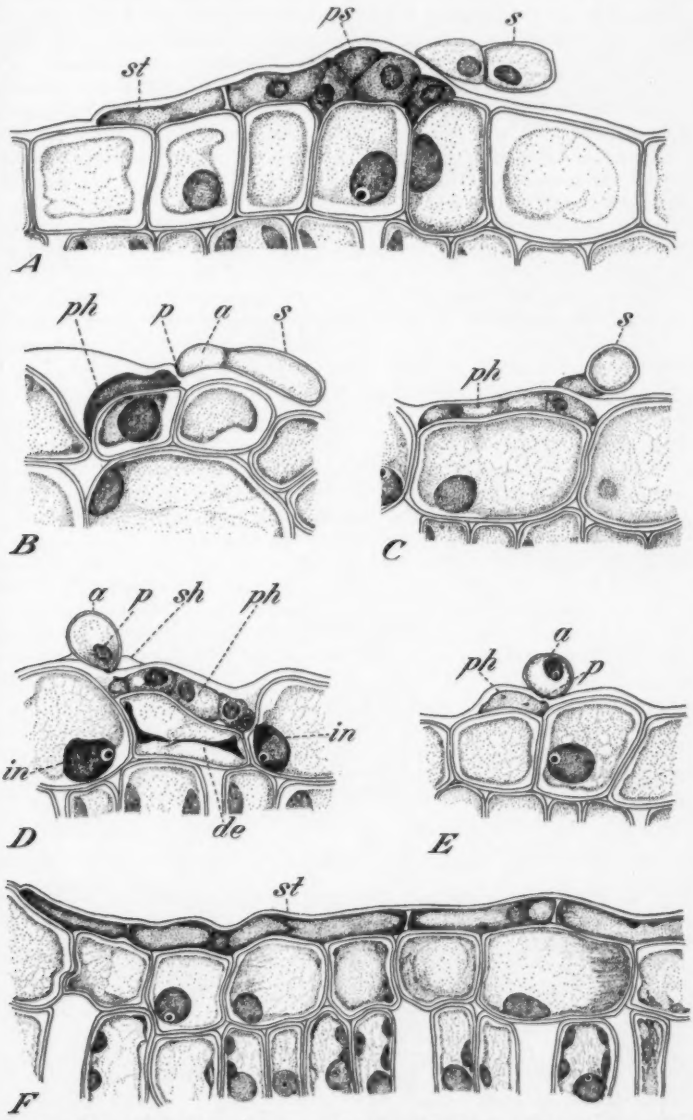

FIGURE 3.—The development of the early stages of infection: *A*, Isolate 22 on Yellow Transparent 2 days after inoculation; spore (*s*), primary stroma (*ps*), stolonlike hypha (*st*); underlying epidermal cells normal. *B*, Isolate 17 on Fameuse 24 hours after inoculation, showing hair-socket infection; spore (*s*), appressorium (*a*), pore (*p*), primary hypha (*ph*). *C*, Isolate 17 on Missouri Pippin 2 days after inoculation; spore (*s*), appressorium lost in sectioning, primary hypha (*ph*). *D*, Isolate 22 on Missouri Pippin 2 days after inoculation; appressorium (*a*), mucilaginous sheath (*sh*), pore (*p*), primary hypha (*ph*), epidermal cell collapsed at *de*, nuclei in adjacent cells at *in* injured. *E*, Isolate 22 on Yellow Transparent 24 hours after inoculation; appressorium (*a*), pore (*p*), primary hypha (*ph*). F, Isolate 22 on Yellow Transparent; stolonlike hyphal branch (*st*) at the periphery of a 6-day-old infection. All × 1000.

Although the underlying palisade cells showed very little depletion, they were apparently injured. The plastids remained and no abnormal vacuolation appeared. As indicated in the upper palisade layer of plate 3, *E*, and shown in detail in the drawing taken from a 16-day-old infection in figure 4, *E*, *ci*, aggregates of small globules appeared in the cytoplasm. These intracellular bodies have been found commonly in certain plant cells parasitized by various fungi and also in cells normally senescent. It is generally believed that they are probably disintegrative products of host cells (*24, p. 37*).

In the central portions of older lesions, where the mycelium was oldest, injured and sometimes dead fungal cells were found. In figure 4, *E*, the fungous cells, *ih* had become highly vacuolate and the one at *dh* was dead, whereas the cells *h* were apparently still robust and healthy. Observations of many similar cases lead to the interpretation that the hyphae live for some time directly superimposed upon dead epidermal cells, but finally they become injured and die.

Isolate 17

The young leaves of Missouri Pippin were decidedly unfavorable for isolate 17. The primary stroma developed very feebly, as shown in a 2-day-old infection in figure 3, *C*. The fungus, however, was able to push out long slender hyphal branches which sometimes involved an area of leaf surface 6 mm in diameter in one lesion. These hyphae were but sparsely branched, and robust secondary stromata were not found. A cross section of one of these hyphal strands near the center of a 16-day-old infection is shown in plate 3, *D*. The strand (*str*) is made up of about four hyphae and each cell appears to be empty. A longitudinal section of a hyphal strand from a 24-day-old infection is shown in plate 3, *A*. The fungal cells (*str*) are sparsely septate and nearly empty, except for inconspicuous nuclei. A drawing (fig. 4, *C*) taken from near the center of a 24-day-old infection shows the details of these host-parasite relations. The hyphal cells (*str*) are long and slender, and contain inconspicuous nuclei (*n*). The underlying host tissues here, as well as those shown in the photomicrographs, are apparently unharmed.

Relationship of the Parasite to the Epidermal Membranes of the Host

The differential staining of the cuticle and the outer wall of the epidermal cell afforded a means of studying the relationship of the apple scab parasite to these membranes. By treating fixed, sectioned material first with Delafield's haematoxylin and then with Sudan IV and mounting in glycerin, the walls of the host cells and of the fungus

Explanatory Legend for Figure 4

Figure 4.—Certain host-parasite reactions: *A*, Isolate 22 on Yellow Transparent 16 days after inoculation; advancing stolonlike hypha (*st*) cleaving its way between the cuticle (*c*) and epidermal cell wall (*w*), the fungus closely adherent to the epidermal cell wall even over the junction of two cells (*j*) where the cuticle is thickest. *B*, Isolate 22 on Yellow Transparent 16 days after inoculation; stolonlike hyphal branch (*st*) near the periphery of the lesion closely adherent to the epidermal cell wall even over the junctions of epidermal cells at *j* where the cuticle is thickest. *C*, Isolate 17 on Missouri Pippin 24 days after inoculation; cells of the stroma (*str*) in the central part of the lesion nearly empty except for small nuclei (*n*); underlying host tissues normal. *D*, *F*, *G*. Isolate 22 on Yellow Transparent 24 days after inoculation, showing successive stages of piercing of cuticle by conidiophores; cuticle (*c*), epidermal cell wall (*w*), conidiophore (*cp*), stroma (*str*), closely fitting collar (*cl*) formed by pierced cuticle about the base of the conidiophore. *E*, Isolate 22 on Missouri Pippin 16 days after inoculation; collapsed epidermal cells (*dc*) underlying a strand of fungus near the center of the lesion, dead fungal cell (*dh*), injured fungal cell (*ih*), apparently healthy cells (*h*), palisade cells slightly impoverished and showing globular inclusions at *ci*. *H*, Isolate 17 on Fameuse 30 days after inoculation; gaps (*g*) in the fungal stroma (*str*) bridged by the cuticle, impoverishment of underlying palisade cells shown by depleted plastids (*dp*) and thin cytoplasm (*cy*). All × 1,060.

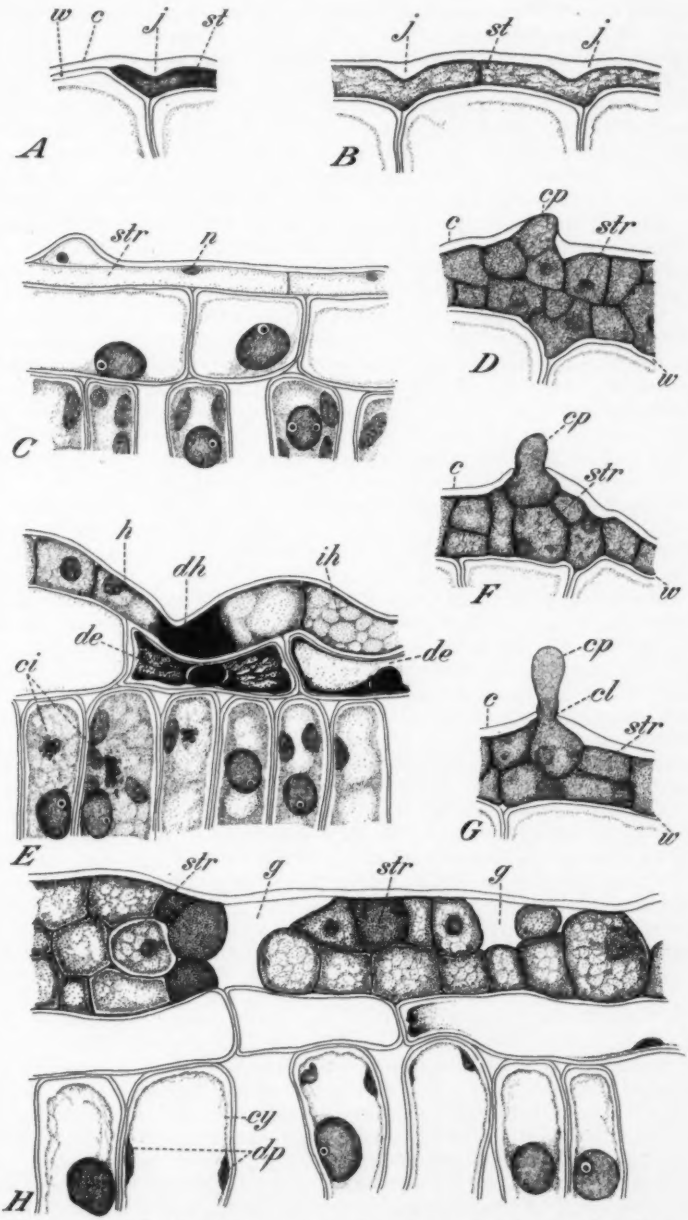

FIGURE 4.—Certain host-parasite reactions. (For explanatory legend see opposite page.)

stained bright blue, whereas the cuticle, being fatty in nature, stained red. The line of demarcation between the cuticle and the epidermal cell walls, in older leaves and over portions of the leaf where the cuticle was relatively thick, was quite sharp. In younger leaves the cuticle was decidedly thinner and was underlaid by a very thin layer that stained reddish purple, indicating that it contained a mixture of fatty and cellulosic materials. This subcuticular layer was sharply set off from the pure blue of the cellulose wall of the epidermal cells but graded off more gradually into the red of the cuticle proper. In older leaves the cuticle always was thickest over the junction of epidermal cells. It was too thin to permit accurate measurements. In the material studied there was no evidence of significant varietal differences in its thickness.

The fungus lay under the cuticle and in intimate contact with the cellulose walls of the epidermal cells. When advancing hyphae were viewed longitudinally, their tips appeared to be wedging or cleaving the cuticle from the epidermal cell wall. Even when passing over the juncture of two epidermal cells the hyphae dipped below the thickened cuticle. These phenomena are illustrated in two drawings made at and near the periphery of a 16-day-old lesion (fig. 4, *A*, *B*). Rarely, and only in preparations of the isolate 22-Missouri Pippin combination, has the fungus been observed to pass through these dentate thickenings of the cuticle. In such cases very small masses of red-staining material appear below the hyphae at the junction of cells.

Even in very old infections where the fungal stroma had become quite thick, the cuticle remained intact, except where conidiophores ruptured it, and acted as a protective covering for the fungus. In order to function in this manner, the cuticle must possess a high degree of elasticity and tensile strength. Indirect evidence to demonstrate these qualities is seen in figure 4, *H*, and the three drawings showing details of the way in which the conidiophores rupture the cuticle (fig. 4, *D*, *F*, *G*). In *H* the increase in size of the fungous cells created such a stress in the area between different stromata that the cuticle was locally ripped from the epidermal cell walls. Consequently, the cuticle bridged the gaps shown at *g*. This phenomenon was very common in older infections in aggressively parasitic isolate-variety combinations. In figure 4, *D*, an expanding conidiophore is stretching the cuticle outward. In *F* and *G* the cuticle has finally been penetrated and the conidiophore is free. The perforated cuticle, however, forms a closely fitting collar about the neck of the conidiophore at *cl*.

DISCUSSION

The results of the present investigation as they relate to spore germination and penetration of the parasite to the subcuticular position are in general agreement with those of Aderhold (*1*, *2*) and Wiltshire (*33*), though they differ in certain particulars. The emanation, in some cases, of the infection hypha from a germ tube or spore without the formation of a characteristically differentiated appressorium has been described in an earlier section of this paper (*14*). The development of the characteristic circular thickening and pore in the basal wall of the appressorium or its equivalent, as herein described, seems not to have been reported by the earlier investigators. In size

and form, the infection hyphae observed by the present writers differ much from those figured by Wiltshire (*33*), being so slender that they were very near the limit of visibility under the oil-immersion lens at a magnification of 1,425 diameters. These slender infection hyphae are very similar to those of *Gymnosporangium juniperi-virginianae* (*18*) and *Puccinia graminis* (*29*). While the possibility of occurrence of a minutely local solvent action on the cuticle by materials emanating from the fungus can scarcely be disproved by the technique employed, the present investigation yielded no convincing evidence that *Venturia inaequalis* exerts a solvent effect on the cuticle of its host. This finding is at variance with the conclusions of Wiltshire. There would seem to be greater probability of a similar action on the subcuticular layer, but the preparations studied by the writers did not show convincing evidence on this question.

As was pointed out in the introduction, parasitism of *Venturia inaequalis* is very distinctive, yet strikingly similar in many respects to that of many obligate parasites, such as certain of the rust and powdery mildew fungi. Notwithstanding the fact that the leaf-invading mycelium in its parasitic phase is limited to the subcuticular position, the fungus is able to derive its nourishment from the underlying host tissues so efficiently that it can develop a very substantial thallus and produce conidia abundantly. It is capable of inciting profound changes in host tissues many cells removed, in some cases leading to their death. As is common among the higher parasites, the species *V. inaequalis* is made up of many variant biotypes (*19*, *20*, *25*, *26*, *30*) with different pathogenic capabilities, some of which are able to live for many weeks in intimately balanced relations with the living tissues of a congenial host. Although in its parasitic phase the fungus is usually limited to subcuticular invasion, the efficiency of its nutrition seems to compare favorably with that of many fungi that freely invade the intercellular spaces or the living cells of the host. The mycelium in close contact with the cellulose walls of the epidermal cells is evidently very efficient in gaining nourishment through them. The subcuticular invaders of the type of *V. inaequalis* may therefore be regarded as a distinctive group among the higher parasites. The fact that they can readily be cultured in vitro and that at least one of them can be controlled genetically by breeding technique (*16*) makes them attractive subjects for further studies in the field of parasitism and disease resistance.

Though the cytological technique does not afford a basis for determination of the nature of resistance of apple varieties to *Venturia inaequalis*, it contributes to the foundation for further studies of this problem. It is noteworthy that spore germination and penetration of the cuticle of young leaves occurred freely, without reference to the isolate or the host variety used. These results, which are in general agreement with the work of Wiltshire (*33*), Johnstone (*9*), and Schmidt (*26*), indicate that resistance in young apple leaves is attributable primarily to relations of the fungus and materials emanating from the host tissues, rather than to mechanical barriers. Some possibilities as to the nature of similar host-parasite relations have been discussed by one of the writers (*18*, p. 591). The fact that substantially different reactions occurred when different incompatible host-isolate combinations were employed in the present work suggests the need for more

extended studies before these reactions can be satisfactorily characterized. It is noteworthy that in one of the combinations studied there was clear evidence of hypersensitiveness of the host, resulting in local necrosis of epidermal cells, while in the other, with another isolate on the same host, there was no evidence of hypersensitiveness.

The present study, in which two monoconidial isolates were employed on three apple varieties, has included four distinct types of host-parasite reaction. The observed variations in reaction are thought to be due partly to differences in the degree and partly to differences in the kind of phenomena concerned. It is to be expected that use of a wider range of biotypes of host and parasite would increase the range of variation in host-parasite relations. The knowledge that *Venturia inaequalis* is heterothallic and the availability of technique for breeding it (*17*) point the way to further studies of host-parasite relations, with genetically controlled isolates. Such work is in progress.

SUMMARY

The host-parasite relations of two monoconidial isolates of *Venturia inaequalis* on the leaves of three apple (*Malus sylvestris*) varieties were studied by cleared-leaf and cytological techniques. The six isolate-variety combinations employed showed four distinct types of host reaction, very susceptible, intermediate, and two types of resistant.

Spore germination, formation of appressoria or functionally equivalent structures, and direct penetration of the cuticle occurred in all cases, without being perceptibly influenced by isolate-variety combinations. A very tenuous infection hypha emanated from a minute pore surrounded by a circular thickening in the basal wall of the appressorium or its functional equivalent (sometimes a spore or germ tube), and pierced the cuticle without visible change in the thickness or staining reaction of the latter. The distal end enlarged to form a primary hypha when it reached the cellulose wall, and the fungus developed beneath the cuticle in close contact with the outer walls of the epidermal cells.

In very susceptible leaves host cells showed little abnormality until about 10 days after inoculation. Then progressive depletion of plastids and cytoplasm, attended by increasing vacuolation, began to appear in the upper palisade region at the center of the lesion. This impoverishment gradually spread throughout the area underlying the fungus, and was followed by necrosis. The fungus showed no apparent injury until the host cells had died. In leaves of intermediate susceptibility, the fungus developed less vigorously, impoverishment of the host cells was less rapid and severe, and little necrosis occurred. In resistant leaves, growth of the fungus was sharply restricted, the resistant variety employed showing hypersensitiveness to one isolate but not to the other. The resistance of young leaves is attributed primarily to relations of the fungus and materials emanating from the host tissues, rather than to mechanical barriers.

It is shown that in suitable isolate-variety combinations the fungus, though confined to the subcuticular position, is able to derive its nourishment efficiently from the underlying host tissues and to incite a wide range of pathological effects therein.

In its mode of penetration of the cuticle, diversity of isolate-variety reactions, and ability to live efficiently for many weeks in intimately

balanced relations with the living cells of a congenial host, *Venturia inaequalis* is considered to be strikingly similar to many of the so-called obligate parasites, and the subcuticular invaders of its type are regarded as a distinctive group among the higher parasites.

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DETERMINING THE SCLEROTIAL POPULATION OF SCLEROTIUM ROLFSII BY SOIL ANALYSIS AND PREDICTING LOSSES OF SUGAR BEETS ON THE BASIS OF THESE ANALYSES [1]

By L. D. LEACH, *assistant plant pathologist*, and A. E. DAVEY, formerly *associate in plant pathology, California Agricultural Experiment Station* [2]

INTRODUCTION

With most soil-borne plant pathogens it has been extremely difficult to determine the extent of survivial in commercial fields except by planting a susceptible crop and measuring the percentage of infection. The appearance of sclerotia as a stage in the life cycle of certain fungi provides a unit of the pathogen which can readily be separated from soils. King and Hope [3] have taken advantage of this fact to study the distribution of sclerotia of *Phymatotrichum omnivorum* (Shear) Duggar by washing samples of soil, taken with a soil auger, through screens of appropriate sizes. Rogers [4] constructed special equipment for the recovery of sclerotia of the same fungus from large soil samples.

DETERMINATION OF SCLEROTIAL POPULATION

ANALYSIS OF SOIL SAMPLES

In the summer of 1932 it was determined [5] that sclerotia of *Sclerotium rolfsii* Sacc. could be recovered from sugar-beet field soils by washing samples through a series of three soil screens of 10, 20, and 40 meshes to the inch. All sclerotia passed through the 10-mesh screen except occasional aggregates, which are rarely found in soil samples. The majority of sclerotia formed on favorable hosts were retained in the 20-mesh screen, but in certain samples large numbers were recovered on the 40-mesh screen. After the finer soil particles were washed through the screens the residue from each screen was flushed into a large white porcelain pan with sufficient water to make a depth of 2 cm. When this container was placed under a strong light it was comparatively easy to separate sclerotia from weed seeds and other extraneous material and to remove them with forceps.

To determine viability, the sclerotia recovered in this manner were surface-sterilized by immersion in bichloride of mercury 1–1,000 for 45 seconds, washed in sterile water, and then plated on potato dextrose agar and incubated at 30° C. The rapid growth of the fungus

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[2] The writers wish to express their appreciation to M. W. Gardner, plant pathologist and J. B. Kendrick, associate plant pathologist, University of California, for suggestions and criticism during these investigations.

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mycelium necessitated the aseptic removal of blocks of agar surrounding germinated sclerotia at intervals of from 12 to 24 hours to prevent overgrowth of other sclerotia on the same plate. With some lots of sclerotia evidence was obtained that the bichloride of mercury was absorbed by sclerotia in amounts sufficient to prevent their germination.[6] In spite of the surface sterilization and ordinary laboratory precautions, contaminating fungi or bacteria from the interior of the sclerotia or from the air frequently interfered with the germination of sclerotia.

To eliminate these difficulties a revised method of determining viability has been used in all recent trials. Sclerotia are plated without chemical treatment on the surface of finely screened, unsterilized peat soil in Petri dishes. The soil is then thoroughly moistened and incubated at 30° C. for 5 days. The mycelium of *Sclerotium rolfsii* can be readily identified on the background of black soil (fig. 1). Germinating sclerotia and the mycelial colonies originating therefrom are removed from the surface of the plates each day during the incubation period.

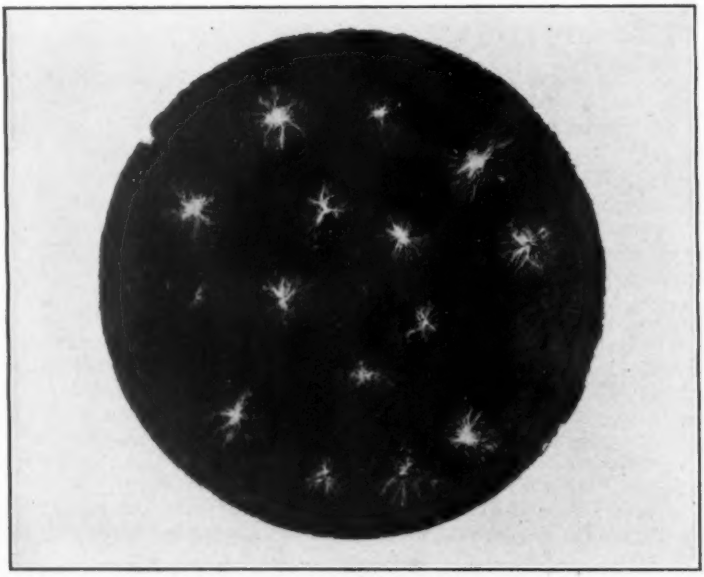

FIGURE 1.—Sclerotia on unsterilized peat soil after 30 hours' incubation at 30° C. Observe the well-developed mycelial colonies.

DISTRIBUTION OF SCLEROTIA IN SOILS

Studies on the distribution of sclerotia were undertaken as soon as the method of removing sclerotia had been perfected. This was done by excavation and observation in the fields and by counts of sclerotia recovered from soil samples representing different horizons.

It was determined that sclerotia may be formed on radiating

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hyphae at a distance of 20 inches or more from infected sugar beets (*Beta vulgaris* L.), but that the largest number occurred within 4 or 5 inches of the beet. From the soil samples studied it was estimated that the fungus in consuming a fully grown beet often produces between 10,000 and 20,000 sclerotia. The vertical distribution of sclerotia in the soil near infected sugar beets is shown in table 1.

TABLE 1.—*Vertical distribution of sclerotia in soil of sugar-beet fields*

Depth (inches)	Sclerotia per 200 g of soil			
	Field A		Field B	
	Number [1]	*Percent*	*Number* [1]	*Percent*
0–3	200	47.5	93	35.3
3–6	143	33.9	126	47.9
6–12	73	17.3	42	16.2
12–18	5	1.1	1	.3
18–24	1	.2	1	.3

[1] Average of 4 samples.

It is evident from table 1 that about 80 percent of the sclerotia occur in the upper 6 inches of the soil and that relatively small numbers of sclerotia are found below the 12-inch level.

The picture presented by a heavily infested sugar-beet field is that of a large number of small areas each containing an enormous population of sclerotia. These areas often coalesce because of the spread of mycelium from originally infected beets to adjacent plants. On the other hand, a small heavily populated area may be surrounded by an area containing few if any sclerotia. As the soil is disturbed by irrigation and cultural operations, the sclerotia gradually become more uniformly distributed. A certain degree of nonuniformity of distribution is maintained, however, by infection and multiplication of the fungus upon weeds or cultivated hosts.

METHOD OF SAMPLING INFESTED AREAS

During these investigations the plan for selecting soil samples has been modified as the work progressed. After several preliminary trials a permanently located plot 300 feet square (approximately 2 acres) was selected as a unit of area for the purpose of following the fluctuation of sclerotial population over a period of years. This area was sufficiently large to reduce the effect of small, severely infested spots. According to the first plan followed, soil samples were collected from nine stations equally spaced on the diagonals of the square. At each station, five cores were taken to a depth of 8 inches with a ⅞-inch soil tube. This method, although providing a picture of the irregular distribution of sclerotia, did not furnish a sufficient number of soil cores to provide an accurate estimate of the population of sclerotia. An improvement was made by collecting 10 soiltube cores in a circle with a radius of 10 feet at each of the 9 stations (fig. 2). The 10 cores from each station were washed through the screens as a composite sample.

When the main purpose is to determine the average survival of sclerotia within a given portion of the field it is possible to save con-

siderable time and effort with little loss of efficiency by combining all the samples collected from a given plot. After the soil is dried, pulverized, and thoroughly mixed it is passed through a Boerner sampler and four aliquot samples of 200, 400, or 800 g, depending on the degree of infestation, are selected. These four samples are washed through screens separately and the recovered sclerotia are plated to determine the percentage of germination.

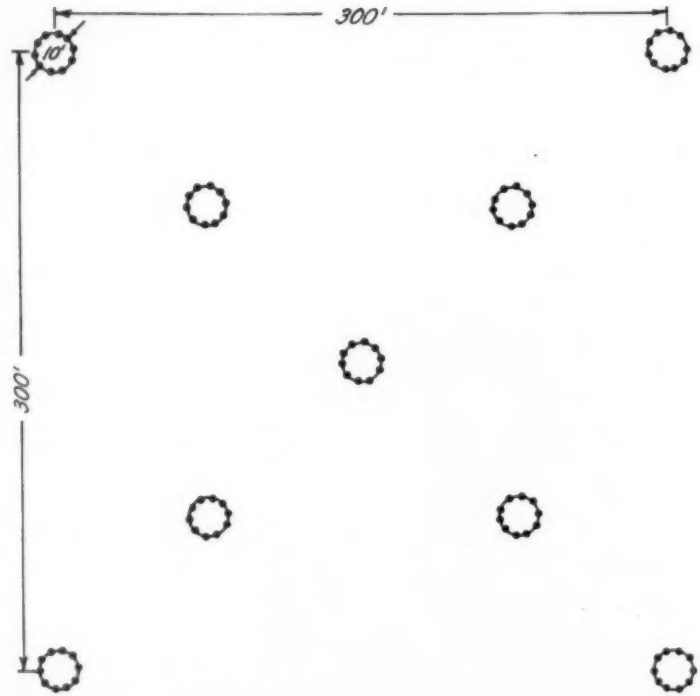

FIGURE 2.—Distribution of soil samples in a permanently located area; 10 soil cores are collected at each of the 9 stations.

TESTING THE ADEQUACY OF THE METHOD

The number of sclerotia obtained from the nine individual stations in several fields are shown in table 2. It would be highly desirable to apply statistical methods to the data and thus determine the reliability of the results. However, it can be readily seen that the distribution of numbers of sclerotia found in the individual samples is not normal and it is doubtful whether one is justified in analyzing such data statistically. In spite of this objection the standard error of the mean and the coefficient of variation have been determined for data from several fields.

TABLE 2.—*Number of sclerotia recovered in 1932 and 1934 from 200-g samples of soil in commercial fields*

Station No.	Field A, 1932 [1]	Field G, 1932	Field H, 1934	Field F, 1932	Field F, 1934
	Number	*Number*	*Number*	*Number*	*Number*
1	125	65	6	40	2
2	0	2	9	2	1
3	40	41	12	1	2
4	24	1	2	20	8
5	58	2	3	13	1
6	165	6	8	0	4
7	30	28	12	16	6
8	38	3	16	19	2
9	340	4	11	0	1
Mean	91.1	16.9	8.8	12.3	3.0
S. E. of mean	36.5	7.6	1.6	4.5	.9
Period elapsed since last beet crop	*Years* 0	*Years* 1	*Years* 1	*Years* 1	*Years* 3
Loss in last crop of sugar beets	*Percent* 70	*Percent* 75	*Percent* 50	*Percent* 50	*Percent* 50

[1] Number of sclerotia recovered from 200 g of air-dry soil.

TABLE 3.—*Number of sclerotia recovered from 400-g soil samples collected from 25 stations in sugar-beet fields F, H, and Q in 1934*

Number of sclerotia recovered and approximate position of indicated field-station samples														
Field F					Field H					Field Q				
*2	2	17	14	*3	*17	14	16	9	*13	*1	0	5	0	*1
9	*7	9	*2	3	29	*16	57	*6	43	117	*10	24	*0	0
2	3	*2	11	17	20	8	*23	20	13	12	34	*2	3	0
3	*11	2	*4	3	10	*24	10	*32	36	2	*36	2	*1	2
*4	3	11	42	*17	*24	27	4	51	*3	*6	62	5	3	*0

*Asterisks indicate the 9 samples that would have been collected in using the method illustrated in fig. 2. The first 3 columns under "Field Q" represent data from the part of the field that had been planted to sugar beets the previous year. See figure 3.

On the basis of 25 samples:
Field F: Mean, 8.0±1.86; coefficient of variation, 23.2 percent.
Field H: Mean, 21.4±1.92; coefficient of variation, 9.0 percent.
Field Q: Mean, 13.1±5.26; coefficient of variation, 40.1 percent.

On the basis of 9 samples:
Field F: Mean, 5.8±1.74; coefficient of variation, 30.0 percent.
Field H: Mean, 17.6±3.10; coefficient of variation, 17.6 percent.
Field Q: Mean, 6.3±3.87; coefficient of variation, 61.4 percent.

Standard error rather than probable error is shown after the sign ±.

In three fields the results obtained from 9 stations were compared with those from 25 stations distributed over the same area (table 3). In two of the cases, fields F and H, the infestation was uniformly distributed and the results obtained by taking the larger number of samples did not differ greatly from those obtained by the usual method. In contrast to the uniform distribution of sclerotia in the above-mentioned fields, field Q shows a distinct separation into areas of high and low populations. When the samples were taken a more uniform distribution was expected and the writers were at a loss to explain the results. Subsequently information was obtained which showed that one part of the field had been in sugar beets the previous season while the other part had been planted to a relatively resistant

crop. Figure 3 shows this field just before harvest. The portion of the field on the left suffered a loss of over 75 percent of the crop. (See data in table 3, first three columns under "Field Q"). The portion on the right indicated by soil samples as being lightly infested showed a loss of less than 20 percent at harvest time. The foregoing results indicate that in uniformly infested fields (fields F and H) the use of nine samples provides a fair estimate of the sclerotial population, whereas in a nonuniform field (field Q) the same number of samples is clearly inadequate. The use of 25 samples from each plot would seriously limit the scope of the survey. Permanently located plots have, therefore, been established only in uniformly infested fields, and since 1934 nine samples have been collected from such plots according to the plan shown in figure 2.

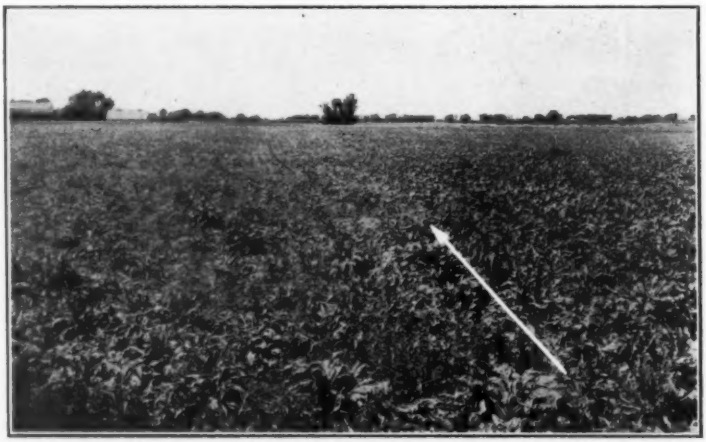

FIGURE 3.—Effect of replanting sugar beets in a field infested with *Sclerotium rolfsii* (field Q in table 3). The part of the field on the left of the arrow was in sugar beets the previous year (1933) and soil samples collected in 1934 before any beets were infected showed over 1,000 living sclerotia per square foot. Over 75-percent loss resulted. The part on the right was not in sugar beets the previous year and showed less than 100 living sclerotia per square foot. Less than 20 percent of the beets were lost by southern sclerotium rot.

NUMERICAL EXPRESSION OF SCLEROTIAL COUNTS

The population of sclerotia in field soils is expressed in this report in terms of sclerotia per square foot in the top 8 inches of soil (two-thirds of a cubic foot) and is derived in the following manner. The number of sclerotia recovered from a set of samples is multiplied by the quotient obtained by dividing the air-dry weight of the soil in grams into 25,100, the approximate weight in grams of two-thirds of a cubic foot of soil when the volume weight is 83 pounds per cubic foot. For example, if a total of 85 sclerotia is recovered from 10 soil samples whose total air-dry weight is 6,275 g, the average population of sclerotia per square foot is determined as follows:

$$85 \text{ (number of sclerotia)} \times \frac{25{,}100}{6{,}275} = 340 \text{ sclerotia per square foot, 8 inches deep.}$$

If the weight of air-dry soil is taken in pounds, the total weight is divided into 55.33 (pounds of soil in two-thirds of a cubic foot); and if in ounces, the total weight is divided into 885 (ounces of soil in two-thirds of a cubic foot).

Examples:

$$85 \text{ (number of sclerotia)} \times \frac{55.33 \text{ pounds}}{13.83 \text{ pounds}} = \frac{340 \text{ sclerotia per square foot,}}{8 \text{ inches deep.}}$$

or

$$85 \text{ (number of sclerotia)} \times \frac{885 \text{ ounces}}{221 \text{ ounces}} = \frac{340 \text{ sclerotia per square foot,}}{8 \text{ inches deep.}}$$

If the soil is considerably heavier or lighter than the standard (83 pounds per cubic foot) a correction can be made by multiplying by a factor more or less than 1.0. The indicated viable population is determined by multiplying the total population by the percentage of germination of the sclerotia as indicated by laboratory tests.

FLUCTUATION OF SCLEROTIAL POPULATIONS IN COMMERCIAL FIELDS

In table 4 are presented the total population of sclerotia and the viable population indicated by successive soil analyses from eight commercial fields over a period of from 2 to 5 years. The standard error of the mean for total population is also shown wherever the individual samples from each of the nine stations were screened separately. All other determinations were made from composites of all the soil collected from a given location. The enormous populations of sclerotia produced by *Sclerotium rolfsii* on sugar beets is illustrated by the results from fields A and B. These determinations indicate that with 70 percent of the beets infected in field A over 250,000,000 living sclerotia per acre were present at harvest time, and a 50-percent infection in field B resulted in the production of over 200,000,000 viable sclerotia per acre.

The effect of moderate populations of sclerotia on the infection of a crop of sugar beets is shown in fields C and D where indicated viable populations of 190 and 140 per square foot, respectively, each resulted in a loss of 20 percent of the sugar-beet crop. The populations of viable sclerotia produced in these two fields by the infestations were 2,190 and 3,120 respectively. The percentage of infection and the resultant population of sclerotia in field A, October 1936, are both comparatively low, but this can be accounted for at least partly as the result of a heavy application of nitrogen fertilizer.

In every case where severely infected crops of sugar beets were followed by nonsusceptible crops such as wheat and barley, or by winter crops such as peas, the sclerotia decreased rapidly by death or disintegration. On the other hand, when beans (susceptible) growing in infested soil were supplied with an abundance of surface irrigation water during the warm summer months the population was maintained, and, in some cases, was increased as shown in field B, November 1933; field E, December 1935; and field F, December 1935. The full effect of the increase was, in some cases, not observed until the second analysis following the bean crop as shown in field A, March 1934 and field C, March 1936.

TABLE 4.—*Indicated population of Sclerotium rolfsii sclerotia from successive soil analyses from eight commercial fields, 1932–36*

Field and time sampled	Crop grown	Total population per square foot	Standard error [1] of mean	Indicated [2] viable population
Field A:		*Number*		*Number*
September 1932	Sugar beets, 70 percent loss	9,840	±3,950	6,630
April 1933	Peas	3,060		1,620
November 1933	Beans	1,590		[3] 870
March 1934	Barley	2,890		1,050
August 1934	do	970		460
March 1935	do	440		190
March 1936	Fallow	390	±40	200
October 1936	Sugar beets, 5 percent loss	640	±180	170
Field B:				
December 1932	Sugar beets, 50 percent loss	7,130		4,810
July 1933	Peas	780		150
November 1933	Beans	1,050		[3] 410
March 1934	Peas	150		90
August 1934	Not known	200		80
July 1935	do	90		70
September 1936	Sugar beets, 5 percent loss	260	±170	190
Field C:				
1932	Sugar beets, 2 percent loss			
May 1933	Sugar beets	310		190
September 1933	Sugar beets, 20 percent loss	2,940		2,190
July 1934	Wheat	690		390
March 1935	Peas	420		350
December 1935	Beans	230		140
March 1936	Peas	350	±70	280
November 1936	Beans	60	±10	50
Field D:				
1933	Sugar beets, 5 percent loss			
1934	Wheat			
March 1935	Fallow	160		140
August 1935	Sugar beets, 20 percent loss	3,940	±1,900	3,120
March 1936	Wheat	2,370	±670	2,340
July 1936	do	1,210	±570	690
Field E:				
1931	Sugar beets, 25 percent loss			
September 1932	Wheat	1,080	±720	590
November 1932	do	1,650	±500	500
July 1933	do	220		60
October 1933	Beans	220		[3] 150
July 1934	Wheat	180		30
December 1935	Beans	490		[3] 370
July 1936	Wheat	570	±70	300
Field F:				
1931	Sugar beets, 50 percent loss			
September 1932	Wheat	2,250	±820	850
November 1932	do	2,240	±750	670
August 1933	do	1,020		300
May 1934	Fallow	500	±110	220
Do	do	360	±110	160
November 1934	Beans	50		40
December 1935	do	150		[3] 90
October 1936	do	200	±50	70
Field G:				
1931	Sugar beets, 75 percent loss			
September 1932	Barley	2,790	±1,250	1,370
July 1933	do	650		210
March 1934	Fallow	120		40
August 1934	Asparagus	0		0
July 1935	do	30		10
July 1936	do	[4] 380	±170	240
Field H:				
1933	Sugar beets, 50 percent loss			
March 1934	Fallow	1,130		650
May 1934	Planted to tomatoes	1,100	±190	920
Do	do	1,340	±120	1,080
March 1935	Fallow	160		90
August 1935	Lettuce	150		60
September 1936	do	100	±30	50

[1] Standard error of the mean for the total population (column 3).
[2] Total population times the percentage of germination.
[3] Some infection observed on beans; crops other than beets and beans showed little or no infection.
[4] Sclerotia produced on buried crowns of water grass.

An unusual case is presented in field G. The high population of sclerotia following a severe infestation on sugar beets was rapidly reduced during successive crops of barley in 1932 and 1933. Asparagus, a nonsusceptible crop, was planted in the spring of 1934, and samples collected in August of that year contained no sclerotia, indicating a very low survival. During 1935 and 1936, however, there occurred a significant increase. Observations showed that the increased population occurred, not upon the asparagus, but upon the decaying crowns of water grass (*Echinochloa crusgalli* (*L.*). Beauv.) which had been buried by cultivation preceding a surface irrigation during warm weather.

The differences between the results from successive sets of samples in the same field are usually comparatively large and in many cases are significant despite the relatively high standard error. Where successive determinations from the same field are available the uniformity of results lends weight to the accuracy of the estimates. Some idea of the differences in estimated population that are due entirely to errors of sampling can be secured by comparing the results from the analyses of September 1932 with those of November in the same year from fields E and F. Duplicate sets of samples collected at the same time are shown for field F, May 1934, and field H, May 1934. In each case the differences were well within the limits of differences required for significance as determined by the standard error computed from the results of individual samples.

The relation that exists between the percentage of infection on sugar beets and the population of viable sclerotia found in fields after harvest is shown in table 5. Determinations from 17 plots during 1934–36 show a correlation coefficient of 0.88 $\pm$0.056. It is apparent that the rate of multiplication is much greater in some fields than in others, but the causes of these differences have not been identified.

TABLE 5.—*Relation between the percentage of infection on sugar beets and the population of sclerotia in the soil after harvest*

Field No.	Infection on sugar beets	Indicated viable population of sclerotia per square foot	Field No.	Infection on sugar beets	Indicated viable population of sclerotia per square foot	Field No.	Infection on sugar beets	Indicated viable population of sclerotia per square foot
	Percent	*Number*		*Percent*	*Number*		*Percent*	*Number*
1	1	30	7	20	2, 195	13	47	1, 680
2	2	250	8	20	3, 110	14	50	4, 830
3	5	200	9	25	1, 880	15	70	6, 650
4	10	280	10	30	1, 190	16	80	7, 030
5	10	360	11	40	1, 505	17	84	4, 680
6	15	630	12	46	2, 195			

PREDICTION OF LOSSES IN SUGAR-BEET FIELDS FROM COUNTS OF SCLEROTIA IN SOIL SAMPLES

Comparisons between the populations of viable sclerotia before planting as determined from soil samples and the percentage of infection occurring in sugar-beet crops had been made by the end of the 1935 season in 19 locations representing a variety of conditions.

There is considerable variation in the results from individual fields, but, in general, the percentage of infection was closely related to the original population of viable sclerotia (table 6). Analysis of the data indicated a correlation coefficient of 0.75 ± 0.10.

During the fall and winter of 1935–36 an attempt was made to determine whether this method of estimating populations of sclerotia could be extended to growers' fields and used as a basis for predicting the percentage of infection in a subsequent crop of sugar beets with reasonable accuracy. Sugar companies collected 354 soil samples from 38 growers' fields totaling 1,561 acres. Each sample consisted of

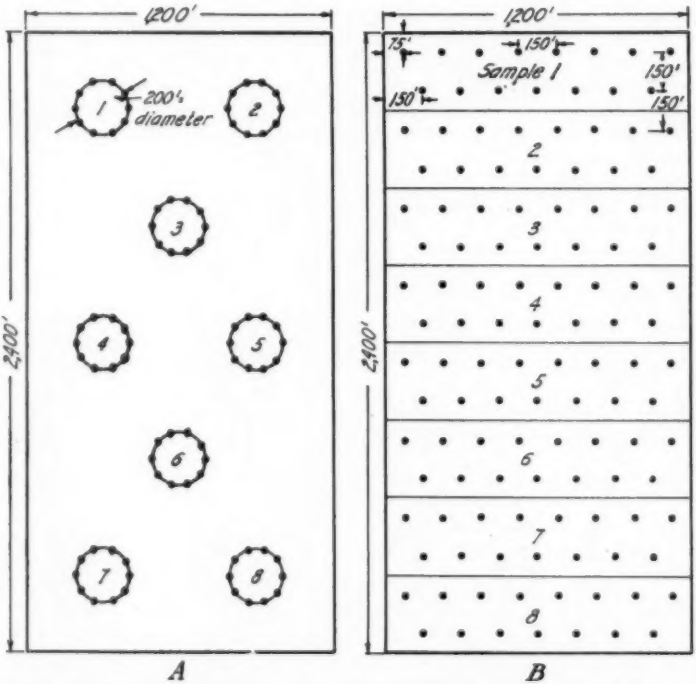

FIGURE 4.—Distribution of soil samples in commercial sugar-beet fields. *A*, Method used in the season of 1935–36. Ten soil-tube cores were collected at each station. *B*, Method used during the season of 1936–37. From 10 to 15 soil-tube cores were combined to form a sample.

10 soil-tube stabs to a depth of 8 inches collected from the perimeter of a circle from 100 to 200 feet in diameter. The sampled areas were uniformly distributed over the entire field intended for planting (fig. 4*A*). These samples were washed separately through screens, the sclerotia recovered, and their viability determined in the laboratories at Davis. Reports covering the indicated population for each sampled field were supplied to the companies. Several fields that had an indicated population of more than 100 viable sclerotia per square foot were rejected for planting to sugar beets for the 1936 season, since previous results had shown that such fields usually suffered a loss of at least 10 percent of the crop.

TABLE 6.—*Relation between the population of sclerotia of Sclerotium rolfsii before planting and the percentage of infection on sugar beets in the indexed area*

[Data collected 1932 to 1935]

Field No.	Indicated viable population of sclerotia per square foot	Infection on sugar beets	Field No.	Indicated viable population of sclerotia per square foot	Infection on sugar beets	Field No.	Indicated viable population of sclerotia per square foot	Infection on sugar beets
	Number	*Percent*		*Number*	*Percent*		*Number*	*Percent*
1	30	10	8	145	20	15	565	40
2	50	4	9	190	25	16	565	64
3	50	8	10	200	20	17	1,380	69
4	70	5	11	215	[1] 42	18	2,070	79
5	75	8	12	220	25	19	2,510	69
6	90	8	13	375	21			
7	95	[1] 35	14	450	50			

[1] Percentage of infection increased by high water table.

Prior to the date of harvest, disease counts were made by examining from 2,000 to 3,000 beets in each area of the field from which a sample was taken. The average percentage of disease for the field was calculated from the sum of all counts in that field.

In table 7 the population of viable sclerotia is shown for each sampled field along with the percentage of infection observed on the date of counting. Because numerous observations have shown that the percentage of infection increases rapidly during July and August, the disease counts have been placed on a comparable basis by converting them to an estimated percentage of infection as of August 1. This was done by applying the rate of increase indicated by an average mortality curve which was secured by counting, at intervals of 10 days or 2 weeks, the increase in number of diseased plants in replicated plots in 12 commercial fields during three growing seasons.

Analysis of the data from commercial fields (table 7) indicates a correlation coefficient of 0.85 ± 0.049 between the indicated population of viable sclerotia (column 2) and the percentage of infection on the subsequent crop (column 3). When the values for percentage of infection were converted to estimates as of August 1, the correlation coefficient (columns 2 and 4) was found to be 0.65 ± 0.10. The percentage of infection in field No. 21 was obviously increased by the excessively high water table. Analysis of the data with field 21 omitted showed a correlation coefficient of 0.86 ± 0.048 between the sclerotial population and the percentage of infection determined by counts (column 3) and of 0.77 ± 0.074 between the sclerotial population and the estimated percentage of infection as of August 1 (column 4).

In the same way the number of sclerotia recovered from each of 293 individual samples was compared with the percentage of infection in the same portion of the field from which the sample was taken. The correlation coefficient was found to be 0.52 ± 0.043 and the standard error of estimate 9.82 percent.

Analysis of the results from individual fields reveals the following information:

(1) As anticipated from previous results, all fields showing less than 100 sclerotia per square foot produced losses of less than 10 percent.

(2) Of the six fields yielding no sclerotia in samples, three showed no infection on beets; the other three showed less than 1 percent infection.

(3) All fields showing more than 200 sclerotia per square foot contained more than 15 percent infection.

(4) The percentages of diseased plants in areas affected by seepage or in those with excessively high water table were much greater than in normal fields with similar populations of sclerotia.

(5) Fields containing localized areas of severe infestations deviated widely (either higher or lower) from the anticipated level of infection.

(6) In two cases a below-average incidence of disease was associated with applications of nitrogen fertilizers.

TABLE 7.—*Relation between fungus populations as determined by analyzing soil samples and the incidence of subsequent infection as measured by field counts, season 1935–36*

Field No.	Viable sclerotia per square foot (average for field)	Infection on subsequent crop	Estimated infection as of Aug. 1	Field No.	Viable sclerotia per square foot (average for field)	Infection on subsequent crop	Estimated infection as of Aug. 1	Field No.	Viable sclerotia per square foot (average for field)	Infection on subsequent crop	Estimated infection as of Aug. 1
	Number	*Percent*	*Percent*		*Number*	*Percent*	*Percent*		*Number*	*Percent*	*Percent*
1	0	0.0	0.0	12	10	[1] 5.2	[1] 7.2	23	40	5.0	7.1
2	0	.0	.0	13	10	[1] 6.9	[1] 4.1	24	80	5.8	11.5
3	0	.0	.0	14	20	2.2	1.3	25	110	11.0	15.4
4	0	.5	1.0	15	20	2.2	1.4	26	130	[2] 3.0	[2] 1.7
5	0	.7	.4	16	30	2.0	1.2	27	140	12.9	9.0
6	0	.8	1.7	17	30	3.1	6.8	28	160	[2] 4.8	[2] 2.8
7	<10	.3	1.1	18	30	[1] 2.7	[1] 1.7	29	350	30.0	21.0
8	<10	.7	.4	19	30	3.3	5.0	30	550	15.4	21.6
9	<10	.7	.6	20	30	[3] 4.5	[3] 2.7	31	950	21.4	15.0
10	10	[1] 3.6	[1] 2.0	21	30	[3] 8.6	[3] 25.8	32	1,190	[1] 32.0	[1] 22.4
11	10	[1] 4.5	[1] 6.4	22	40	1.7	1.4				

[1] Field contained localized areas of high infestation.
[2] Nitrogenous fertilizers applied.
[3] Percentage of infection increased by high-water table.

The results from the individual soil samples showed a lower correlation with the incidence of disease in a particular portion of the field than did the field averages. One-half of the samples yielding no sclerotia were, however, associated with areas showing less than 0.5 percent infection, while three-fourths (15 out of 20) of the samples yielding more than 10 sclerotia (approximately 250 per square foot) were associated with areas showing more than 10 percent infection. In several cases the incidence of disease varied widely from the infestation indicated by the individual sample. This may have been due to error of sampling or to failure to locate accurately the sampled area when disease counts were made.

In view of the foregoing results a revision of the method of selecting soil samples was instituted during the season of 1936–37. Instead of collecting 10 soil-tube stabs from each of 10 or more portions of the field (fig. 4, *A*) single stabs are taken in straight rows across the field at intervals of 40, 50, or 70 yards depending on the intensity of sampling desired. The soil from 10 to 15 stabs (approximately 3 pounds) is combined to form a single sample and is screened as a unit. By staggering the location of the stabs in adjacent rows, a very complete sampling of a field can be secured (fig. 4, *B*). This method may tend to obscure the occurrence of small localized areas of infestation but is expected to greatly improve the reliability of the field average.

SUMMARY

A reasonably accurate approximation of the population of sclerotia of *Sclerotium rolfsii* can be secured by the examination of soil samples collected from growers' fields. The soil samples are washed through a series of three screens of 10, 20, and 40 meshes to the inch. Sclerotia are recovered from the residue and their germinability determined by incubation at 30° C. on the surface of unsterilized peat soil in Petri dishes.

In sugar-beet fields approximately 80 percent of the sclerotia occur in the upper 6 inches of soil and less than 2 percent of the sclerotia are more than 12 inches deep. Soil samples are, therefore, taken to a depth of 8 inches in undisturbed soils.

The population is expressed as the number of viable sclerotia per square foot of soil to a depth of 8 inches, the depth of sampling, and is computed by methods described in this paper. The information obtained in this manner provides a means of estimating the survival of *S. rolfsii* under different crop rotations and cultural conditions.

The fluctuation of sclerotial population has been followed in permanently located areas within 17 growers' fields over periods of from 2 to 5 years. Results from eight of these fields are presented. In each area 10 soil-tube cores were collected at each of nine stations on the diagonals of a square approximately 300 feet on a side.

Results indicate a relatively high correlation between the population of viable sclerotia, determined before planting, and the percentage of infected sugar beets in the same area. The number of sclerotia in the soil increased in proportion to the percentage of infection. Following 50 percent infection of beets, as many as 5,000 sclerotia per square foot have been found. Cropping infested fields with nonsusceptible crops such as wheat or barley, or with winter crops such as peas, results in rapid reduction of the sclerotial population. Susceptible crops, such as beans, when supplied with abundant surface irrigation during the summer months usually maintain the sclerotial population at a moderately high level.

During the seasons of 1932 to 1936 soil samples were collected from 51 fields or portions of fields that were subsequently planted to sugar beets. Fields with more than 200 viable sclerotia per square foot invariably showed more than 15 percent loss of sugar beets, while those with less than 100 per square foot usually showed less than 10 percent loss. This method of estimating the sclerotial population may be used to eliminate severely infested fields in advance of planting.

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